LUNG DEVELOPMENT IN CONGENITAL DIAPHRAGMATIC HERNIA:

An experimental study in a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia induced by Nitrofen

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LUNG DEVELOPMENT IN CONGENITAL DIAPHRAGMATIC HERNIA:

An experimental study in a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia induced by Nitrofen

(Longontwikkeling bij congenitale hernia diaphragmatica)

PROEFSCHRIFT
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Geboren te Hillegom

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Voor de Kindergeneeskunde
Aan Jan en Jelmer

CONTENTS

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Chapter 1

INTRODUCTION

INTRODUCTION

1.1 General Introduction

Approximately 3% of human neonates are born with one or multiple congenital malformations (Nadler 1986). The birth of a child with a physical or mental handicap presents considerable problems with which both parents and the child must cope and raises questions about the cause. Despite our increasing knowledge of genetics and embryology, many times the answers cannot be given; the etiology of the majority of malformations is still a maze of unknowns (table; McKusick 1992; Wilson 1977).

Causes of malformations in man		
Known genetic transmission	20	%
Chromosomal aberration	3-5	%
Environmental causes:		
- radiation	< 1	%
- infections	2-3	%
- maternal metabolic imbalance	1-2	%
- drugs and environmental chemicals	4-6	%
Unknown	65-70	%

Fortunately, the mortality rate of many congenital malformations has dropped continuously over the last years thanks to the development of antenatal diagnostic procedures, advanced surgical techniques, and perioperative intensive care.
In contrast however, the mortality rate of congenital diaphragmatic hernia (CDH) has not improved or changed in the last 30 years, despite antenatal diagnosis and tremendous efforts by pediatric surgeons and pediatric intensivists. CDH is a birth defect in which the organs of the abdominal cavity herniate into the chest cavity through an incompletely closed diaphragm, accompanied by unilateral or bilateral pulmonary hypoplasia with resultant respiratory failure after birth in the majority of the cases. The incidence of CDH is 1:3000 liveborns and the etiology is unknown. In 50% of the cases CDH is associated

with a variety of chromosomal, genetic, and nongenetic defects, but the cause of the absent closure of the diaphragm is still categorized as unknown (Smith 1984).
CDH is a major problem in pediatric surgery due to the severe respiratory insufficiency that may occur immediately after birth, resulting in a mortality rate of 30-50% (Molenaar et al. 1991). The outcome depends mainly on the gravity of pulmonary hypoplasia or on the combination of pulmonary hypoplasia with persistent pulmonary hypertension (Tibboel et al. 1993). The diaphragmatic defect itself is hardly a problem any more; it can be closed surgically. The abnormal development of the lungs is the cause of the high morbidity and mortality rates.
Therefore, we were interested in the pathogenetic aspects of pulmonary hypoplasia, and the following specific questions were formulated and studied in a rat model of CDH and pulmonary hypoplasia induced by 2,4-dichlorophenyl-p-nitrophenyl (Nitrofen):

* Is the development of the lung parenchyma different from normal? If so, in what respect:
  - Is the architecture of the air spaces abnormal?
  - Is the differentiation of the epithelial lining of the air spaces affected?
  - Is the growth pattern of the lung primordium changed?
  - Is the extracellular matrix around the developing lung primordium different in composition?
* Is the closing process of the diaphragm different in case of CDH? If so, in what way?

In addition we asked the question:

* How does Nitrofen, the compound we use to induce CDH in the rat, exert its teratogenic effects?

Before these questions were addressed, a study of the literature was undertaken to assess what is known about normal and abnormal development of the lung.

1.2 **Normal development of the lung**

The onset of pulmonary development takes place in humans at 3-4 weeks, and in the rat on day 11 (Ten Have-Opbroek 1981; Ten Have-Opbroek and Plopper 1992). At that time the pulmonary primordium (Fig. 1) appears as a protrusion of the foregut and proliferates into two lung buds. Each of these lung buds gives rise to a branching tubular system, i.e., the primordial system of the prospective right or left lung (onset of the pseudoglandular phase; Ten Have-Opbroek 1981). These primordial tubules are lined by undifferentiated columnar epithelium. Differentiation of the primordial system into the prospective bronchial system and the prospective respiratory system (unit: pulmonary acinus) starts at 10-12 weeks in humans (Otto-Verberne et al. 1988), and around day 16 in the rat (Otto-Verberne and Ten Have-Opbroek 1987). The epithelium of the prospective bronchial system is columnar, whereas that of the pulmonary acinus is

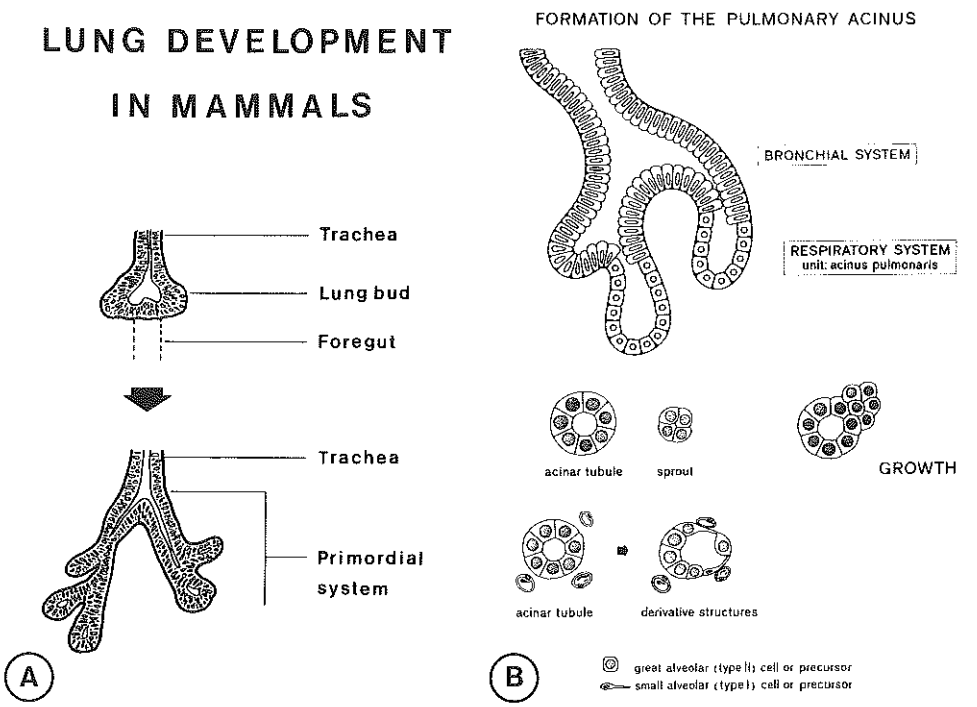

Figure 1 *Diagrams of mammalian lung development (Ten Have-Opbroek, 1981)* ***(A)*** *The two lung buds arising from the primitive foregut (in the rat by day 11) develop into the primordial systems of the right and left lungs. The undifferentiated epithelium lining the primordial tubules is pseudostratified columnar.* ***(B)*** *The prospective bronchial system and the prospective respiratory system (unit: pulmonary acinus) differentiate from the primordial system, in the rat by day 16. The basic structure in pulmonary acinus formation is the acinar tubule or sprout (lining: type II cells). Transformation of acinar tubules results in derivative structures (dilated tubules, saccules, alveoli), which contain not only cuboid type II cells in their lining, but also thinner (developing) type I cells (in the rat as of day 20).*

initially approximately cuboid (Ten Have-Opbroek 1981). By terminal and lateral budding there is an increase in the number of generations of bronchial and acinar tubules. During further development the pulmonary acinus shows dramatic changes: the acinar tubules transform into derivative structures with a duct-, sac-, or pouch-like shape. These derivative structures are lined by cuboid alveolar type II cells and (from type II cells deriving) thin alveolar type I cells (Ten Have-Opbroek 1981). Light microscopical investigation during pulmonary development reveals the so-called pseudoglandular phase (humans: weeks 3-16; rats: days 11-19), followed by the canalicular phase (humans: weeks 13-25; rats: day 20), the saccular phase (humans: weeks 24-40; rats: days 21-22) and the alveolar phase (postnatally in both humans and rats; Ten Have-Opbroek 1981).

1.3 Abnormal lung development

Abnormal development of human lungs occurs isolated or in association with other anomalies, such as hydrops fetalis, renal anomalies, hernia (including congenital diaphragmatic hernia and omphalocele), skeletal anomalies, and abnormalities of amniotic fluid, e.g. oligohydramnios and polyhydramnios (Nakamura et al. 1992).
In all cases, lung/body weight ratios are significantly lower than in normal individuals. In most cases, including those with diaphragmatic hernia, the number of bronchial branches, radial alveolar counts, and the content of disaturated phosphatidylcholine (one of the components of surfactant, the substance that reduces the surface tension in the air spaces) are decreased, while the air-blood barrier is thicker compared to that in control lungs (Nakamura et al. 1992).
Several animal models have been developed to enable studies of pulmonary hypoplasia. In these models pulmonary hypoplasia was induced using amniotic fluid drainage from fetal guinea pigs (Collins et al. 1986; Moessinger et al. 1986) or rats (Blachford and Thurlbeck 1987), tracheal ligation in fetal rabbits (Carmel et al. 1965) or lambs (Fisk et al. 1991), phrenic nerve section in fetal lambs (Nagai et al. 1988), and operative creation of CDH in rabbits (Ohi et al. 1976) or lambs (Adzick et al. 1985; Glick et al. 1992; Pringle et al. 1984). A disadvantage of most of these models is that a surgical procedure is required, and that such a procedure is not possible before the onset of organogenesis of the lung. In addition, fetal surgery is accompanied by high mortality rates; especially in larger experimental animals this may lead to high costs and ethical problems.
Therefore, our group has used the knowledge of several researchers on the teratogenic effects of the herbicide 2,4-dichlorophenyl-p-nitrophenyl (Nitrofen; Ambrose et al 1971; Iritani 1984; Nakao and Ueki 1987) to develop a rat model for CDH and pulmonary hypoplasia (Kluth et al. 1990; Tenbrinck et al. 1990). When administered to the mother on day 10 or 12 of pregnancy Nitrofen interferes with the closing mechanism of the diaphragm and development of the lungs during organogenesis in the offspring. This model offers several advantages above the before-mentioned models: it is inexpensive, and easily reproducible, with shorter duration of pregnancies, multiparity, and last but not least, the possibility to interfere before the onset of development of the lung primordium and closure of the diaphragm. The mechanism of teratogenesis by Nitrofen is largely unknown, but evidence is accumulating that the effects are mediated via alterations in thyroid hormone status (Gray and Kavlock 1983; Manson 1986; Manson et al. 1984).

1.4 Aim and approach of the present study

Aim of the present study was to investigate

(1) whether there are differences in the development of the lung parenchyma between hypoplastic lungs and normal lungs with respect to architecture, proliferation and differentiation of alveolar epithelial cells, and interaction between epithelium and mesenchyme,
(2) whether the closing process of the diaphragm differs in case of CDH, and
(3) how Nitrofen exerts its teratogenic effects.

For this purpose, the Nitrofen rat model was used. Fetuses of different gestational ages were collected, their lungs were dissected out and studied. Nitrofen-exposed lungs, which are hypoplastic, were compared to normal control lungs.
The first part of this thesis describes the morphological characteristics of normal and Nitrofen-exposed late fetal rat lungs. The alveolar epithelial composition and architecture of the pulmonary acinus were studied by light microscopy, using histology, immunohistochemistry and morphometry (chapter 2). The ultrastructural features of the alveolar epithelial cells were studied by transmission electron microscopy (chapter 3).
Subsequently, the development of the diaphragmatic defect and the hypoplastic lungs in the early embryonic and fetal period is described. The observations were made by scanning electron microscopy (chapter 4) and light microscopy, using histology and immunohistochemistry with antibodies against various differentiation and proliferation antigens (chapter 5).
Evidence is accumulating that the effects of Nitrofen teratogenesis are mediated via alterations in thyroid hormone status. Chapter 6 describes in vitro experiments performed to test the effect of Nitrofen on the T3-receptor binding.
The most important research results so far obtained in our Nitrofen rat model are reviewed and discussed in chapter 7.
Finally, the last chapter of this thesis focusses on the comparability of our rat model for CDH and pulmonary hypoplasia and the human situation (chapter 8).

1.5 References

Adzick NS, Outwater KM, Harrison MR, Davies P, Glick PL, deLorimier AA, Reid LM: Correction of congenital diaphragmatic hernia in utero IV. An early gestational fetal lamb model for pulmonary vascular morphometric analysis. J Pediatr Surg 20:673-680, 1985.

Ambrose AM, Larson PS, Borzelleca JF, Smith RB, Hennigar GR: Toxicologic studies on 2,4-dichlorophenyl-p-nitrophenyl ether. Toxicol Appl Pharmacol 19:263-275, 1971.

Blachford KG, Thurlbeck WM: Lung growth and maturation in experimental oligohydramnios in the rat. Pediatr Pulmonol 3:328-333, 1987.

Carmel JA, Friedman F, Adams FH: Fetal tracheal ligation and lung development. Am J Dis Child 109:452-456, 1965.

Collins MH, Moessinger AC, Kleinerman J, James LS, Blanc WA: Morphometry of hypoplastic fetal guinea pig lungs following amniotic fluid leak. Pediatr Res 20:955-960, 1986.

Fisk NM, Parkes MJ, Moore PJ, Haidar A, Wigglesworth J, Hanson MA: Fetal Breathing During Chronic Lung Liquid Loss Leading to Pulmonary Hypoplasia. Early Hum Dev 27:53-63, 1991.

Glick PL, Stannard VA, Leach CL, Rossman J, Hosada Y, Morin FC, Cooney DR, Allen JE, Holm B: Pathophysiology of congenital diaphragmatic hernia II: the fetal lamb CDH model is surfactant deficient. J Pediatr Surg 27:382-388, 1992.

Gray LE, Kavlock RJ: The effects of the herbicide 2,4-dichlorophenyl-p-nitrophenyl ether (Nit) on serum thyroid hormones in adult female mice. Toxicol Lett 15:231-235, 1983.

Iritani I: Experimental study on embryogenesis of congenital diaphragmatic hernia. Anat Embryol 169:133-139, 1984.

Kluth D, Kangah R, Reich P, Tenbrinck R, Tibboel D, Lambrecht W: Nitrofen-induced diaphragmatic hernias in rats: an animal model. J Pediatr Surg 25:850-854, 1990.

Manson JM: Mechanism of Nitrofen teratogenesis. Environ Health Perspect 70:137-147, 1986.

Manson JM, Brown T, Baldwin DM: Teratogenicity of Nitrofen (2,4,-dichloro-4'-nitrodiphenyl ether) and its effects on thyroid function in the rat. Toxicol Appl Pharmacol 73:323-335, 1984.

McKusick VA: Mendelian inheritance in man. John Hopkins University Press, Baltimore and London 1992.

Moessinger AC, Collins MH, Blanc WA, Rey HR, James LS: Oligohydramnios-induced lung hypoplasia: the influence of timing and duration in gestation. Pediatr Res 20:951-954, 1986.

Molenaar JC, Bos AP, Hazebroek FWJ, Tibboel D: Congenital diaphragmatic hernia, what defect? J Pediatr Surg 26:248-254, 1991.

Nadler HL: Teratology. In: Welch KJ, Randolph JG, Ravitch MM, O'Neill JA, Rowe MJ (eds.), Pediatric Surgery, 4th ed, Chicago London Boca Taton, Year Book Medical Publishers, 1986, pp 11-13.

Nagai A, Thurlbeck WM, Jansen AH, Ioffe S, Chernick V: The effect of chronic biphrenectomy on lung growth and maturation in fetal lambs. Morphologic and morphometric studies. Am Rev Respir Dis 137:167-172, 1988.

Nakamura Y, Harada K, Yamamoto I, Uemura Y, Okamoto K, Fukuda S, Hashimoto T: Human pulmonary hypoplasia: statistical, morphological, morphometric, and biochemical study. Arch Pathol Lab Med 116:635-642, 1992.

Nakao Y, Ueki R: Congenital diaphragmatic hernia induced by Nitrofen in mice and rats: characteristics as animal model and pathogenetic relationship between diaphragmatic hernia and lung hypoplasia. Congen Anom 27:397-417, 1987.

Ohi R, Suzuki H, Kato T, Kasai M: Development of the lung in fetal rabbits with experimental diaphragmatic hernia. J Pediatr Surg 11:955-959, 1976.

Otto-Verberne CJM, Ten Have-Opbroek AAW: Development of the pulmonary acinus in fetal rat lung: a study based on an antiserum recognizing surfactant-associated proteins. Anat Embryol 175:365-373, 1987.

Otto-Verberne CJM, Ten Have-Opbroek AAW, Balkema JJ, Franken C: Detection of the type II cell or its precursor before week 20 of human gestation, using antibodies against surfactant-associated proteins. Anat Embryol 178:29-39, 1988.

Pringle KC, Turner JW, Schofield JC, Soper RT: Creation and repair of diaphragmatic hernia in the fetal lamb: lung development and morphology. J Pediatr Surg 19:131-140, 1984.

Smith DW: Recognizable patterns of human malformation. Philadelphia: WB Saunders Co, 1984.

Tenbrinck R, Tibboel D, Gaillard JLJ, Kluth D, Bos AP, Lachmann B, Molenaar JC: Experimentally induced congenital diaphragmatic hernia in rats. J Pediatr Surg 25:426-429, 1990.

Ten Have-Opbroek AAW: The development of the lung in mammals: an analysis of concepts and findings. Am J Anat 162:201-219, 1981.

Ten Have-Opbroek AAW, Plopper CG: Morphogenetic and Functional Activity of Type II Cells in Early Fetal Rhesus Monkey Lungs - A Comparison Between Primates and Rodents. Anat Rec 234:93-104, 1992.

Tibboel D, Bos AP, Hazebroek FWJ, Lachmann B, Molenaar JC: Changing concepts in the treatment of congenital diaphragmatic hernia. Klin Pädiatr 205:67-70, 1993.
Wilson JG: Teratogenic effects of environmental chemicals. Fed Proc 36:1698-1703, 1977.

Chapter 2

ALVEOLAR EPITHELIAL COMPOSITION AND ARCHITECTURE OF THE LATE FETAL PULMONARY ACINUS

An immunocytochemical and morphometric study in a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia

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ALVEOLAR EPITHELIAL COMPOSITION AND ARCHITECTURE OF THE LATE FETAL PULMONARY ACINUS

An immunocytochemical and morphometric study in a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia

2.1 Summary

Aim of the present study was to compare the architecture and alveolar epithelial cell composition of the pulmonary acinus in hypoplastic and normal fetal rat lungs. For this purpose, we used a rat model of pulmonary hypoplasia in association with congenital diaphragmatic hernia (CDH) induced by Nitrofen (100 mg on day 10 of pregnancy). Sections (5 μm) from lungs of control and Nitrofen-exposed fetal Sprague Dawley rats with or without CDH aged 18-22 days (vaginal plug on day 1, birth on day 23) were stained with hematoxylin and eosin. To identify developing alveolar epithelial cells, sections were incubated with anti-Surfactant Protein A (SP-A; rabbit anti-mouse) or preimmunization serum (indirect immunofluorescence). On days 18 and 19, control lungs and exposed lungs from fetuses with and without CDH looked similar (pseudoglandular stage of lung development). The prospective pulmonary acinus consisted of acinar tubules with small round lumens, lined by cuboid, fluorescent type II cells. Morphometric analysis on day 19 showed significantly smaller lung volumes and lung tissue volumes after Nitrofen-exposure. On day 20 (canalicular stage), some tubules were slightly dilated and lined by cuboid and thinner fluorescent cells; these dilated tubules were less numerous in lungs from exposed fetuses with CDH. On days 21 and 22 (saccular stage), the saccular lining consisted of cuboid to thin fluorescent cells in exposed lungs from fetuses with and without CDH, and fluorescent (low) cuboid cells interspersed with dark zones (type I cell areas) in control lungs. In the exposed lungs from fetuses with CDH, the lumens of all air spaces were frequently slit-like, and the septa were thicker. These phenomena gave the lungs a primitive, compact aspect. Morphometric analysis on day 22 showed smaller lung volumes and lung tissue volumes, smaller air space/tissue ratios, smaller epithelial surface areas, and more type II cells per surface area in Nitrofen exposed lungs than in normal control lungs. We conclude that Nitrofen-exposed, and thus hypoplastic, fetal rat lungs are retarded with respect to the differentiation of cuboid type II cells into squamous type I cells whether or not CDH is present and the development of the future air spaces between days 20 and 22 if CDH is present.

2.2 Introduction

Abnormal development of the lungs occurs isolated or in association with other anomalies. Associated anomalies are congenital diaphragmatic hernia (CDH), or oligohydramnios as found in agenesis or severe dysplasia of the kidneys, and premature rupture of the membranes. CDH is a serious anomaly in humans with unknown etiology and an incidence of 1:3000 newborns. The abnormal pulmonary development leads to pulmonary hypoplasia, which forms one of the main causes of death in CDH. Posterolateral diaphragmatic hernia, known as Bochdalek hernia, accounts for 75 to 85% of the cases and occurs most frequently on the left side. The defect in the diaphragm allows abdominal viscera (liver, bowel loops) to herniate into the thorax. The mortality rate is 40-60% despite progress in prenatal diagnosis and changing concepts in therapeutic approaches (Hazebroek et al. 1988; Wenstrom et al. 1991). The clinical picture and history of CDH has been described in several reviews (Cullen et al. 1985; Puri and Dewan 1989; Molenaar et al. 1991). Histologic studies describing the different stages during abnormal lung development and the resulting morphology in patients with CDH are limited and the results are divergent (Areechon and Reid 1963; Boyden 1972; Geggel et al. 1985; George et al. 1987; Kitagawa et al. 1971; Nakamura et al. 1991; Reale and Esterly 1973). These reports mention a lower number of bronchial branches and alveoli, and increased muscularity of the pulmonary vascular bed.

Several studies on the pathogenesis of pulmonary hypoplasia have been performed in animal models. In general, an important advantage of these animal models is that they allow to study abnormal lung development at a number of prenatal time-points, without any additional damage by mechanical ventilation or medication; a problem frequently encountered in studies with human lung tissue. In almost all experimental studies CDH was created surgically in fetal sheep or rabbits. With the exception of one study (Adzick et al. 1985), surgical intervention was done relatively late in gestation, after closure of the pleuroperitoneal canals and affected late prenatal lung development (Harrison et al. 1985; Ohi et al. 1976; Pringle et al. 1984). For the present study we used a rat model available in our department, in which pulmonary hypoplasia and CDH are induced by the herbicide 2,4-dichlorophenyl-p-nitrophenylether (Nitrofen) administered to the mother on day 10 of pregnancy (Kluth et al. 1990; Tenbrinck et al. 1990; Tibboel et al. 1993). This early administration, 6 days before normal closure of the pleuroperitoneal canals, coincides with the anlage of the lung primordium. Using this model our group and other authors have found smaller lung volumes, lung weights, and lung weight/body weight ratios as well as decreased radial saccular counts (Lau et al. 1988; Stone and Manson 1981; Tenbrinck et al. 1990). These findings meet the criteria of Askenazi and Perlman (1979) for pulmonary hypoplasia in humans, and indicate that the Nitrofen-exposed rat lungs are hypoplastic indeed (Tenbrinck et al. 1990). The classical view is that hypoplastic lungs in CDH result from compression by bowel loops protruding through the diaphragmatic defect. Studies in

the Nitrofen model do not support this view. They have indicated that growth impairment is the result of a competition for space in the embryonic thoracic cavity (Kluth et al. 1993) rather than the result of compression, and that the lung may even be primarily hypoplastic (Iritani 1984; Nakao et al. 1990; Ueki et al. 1990). Human case reports also suggest that hypoplastic lungs are not only smaller, but also immature as reflected by amniotic fluid lecithin/sphingomyelin ratios (Berk and Grundy 1982; Hisanaga et al. 1984). Only one study in the rat model gives some details concerning the alveolar epithelial lining (see discussion; Kimbrough et al. 1974).

Aim of our study was to obtain more information about the developmental process of pulmonary hypoplasia in CDH by studying the architecture and alveolar epithelial composition of the pulmonary acinus in the hypoplastic fetal rat lung at several prenatal time-points (days 18-22) in the presence and absence of CDH. For general orientation, and for morphometry, hematoxylin and eosin-stained paraffin sections were examined by light microscopy. To identify developing type II and type I cells, fresh-frozen and paraffin sections were incubated with anti-Surfactant Protein A antiserum and, after immunofluorescent or enzyme staining, studied with immunofluorescence or conventional light microscopy. The results were compared with our findings in normal fetal rat lungs. For interpretation of the pictures we used well-defined criteria for recognition of fetal type II cells and prospective respiratory structures (Ten Have-Opbroek 1981; 1991).

2.3 **Methods**

Tissues

Pulmonary hypoplasia in association with CDH was induced as described before (Kluth et al. 1990; Tenbrinck et al. 1990): Adult Sprague Dawley rats were mated overnight. The next day, if a vaginal plug was present, was considered to be day 1 of pregnancy. After a short anaesthesia with ether, a single dose of 100 mg Nitrofen dissolved in olive-oil was administered to the mothers through an intragastric tube on day 10 of pregnancy. Pregnant control animals received the same dose of olive-oil without Nitrofen. Fetuses were collected by Caesarean section from day 18 through day 22 (birth on day 23). They were weighed and killed after cervical intersection with a needle or intraperitoneal injection of Nembutal (750 μg), depending on fetal age. Presence, position and size of a diaphragmatic defect were evaluated, and the lungs were dissected out. Each litter was randomly subdivided into four groups. Two of those groups (100 fetuses in total; 43 Nitrofen-exposed, 57 control) were used in this study. The lungs from one group (45% Nitrofen-exposed, 55% control) were fixed by immersion in 3.6% buffered formalin (pH 7.0) at 4°C; those from the other group (42% Nitrofen-exposed, 58% control) were immediately frozen in tissue-tek embedding medium (O.C.T. Compound, Bayer,

Mijdrecht, The Netherlands) as described before (Otto-Verberne and Ten Have-Opbroek 1987) and stored at -20°C.

Antisera

Besides morphologic criteria, anti-Surfactant Protein A (SP-A) antisera were used to recognize both mature and immature type II alveolar epithelial cells (i.e. cells containing multilamellar bodies and their precursory stages vs. precursory stages only) in fetal lungs on the basis of a cytoplasmic staining (see Results). The antisera, previously made by Dr. Ten Have-Opbroek, were available in the laboratory (Oomen et al. 1990; Van Hemert et al. 1986). They had been prepared by injecting a rabbit with bronchoalveolar lavage fluid (30,000 g pellet) or lung homogenate from adult mice. The rabbit sera were absorbed with cross-reacting organs to obtain a lung-specific reaction. The resulting immune sera were called Specific Anti-Lavage Serum (SALS) and Specific Anti-Adult mouse Lung Serum (SAALS). They recognize the 32-35 kD proteins of SP-A when applied to blots of mouse lung lavage proteins (Oomen et al. 1990; Ten Have-Opbroek 1991; Van Hemert et al. 1986), and mark type II cells strongly in several species (Oomen et al. 1990; Otto-Verberne and Ten Have-Opbroek 1987; Otto-Verberne et al. 1988; 1990; Ten Have-Opbroek 1975; 1979; 1981; 1991; Ten Have-Opbroek and Plopper 1992; Van Hemert et al. 1986). To eliminate background fluorescence at early fetal stages (Ten Have-Opbroek 1979), SALS was additionally absorbed with homogenate made from 14 day-old-rat fetuses (SALS-E). This absorption does not affect the type II cell reactivity of the antiserum SALS (Oomen et al. 1990), because rat lungs at the age of 14 days contain only primordial epithelium (Otto-Verberne and Ten Have-Opbroek 1987). Preimmunization serum (PS) was used as control.

Staining procedures

The formalin-fixed tissue was dehydrated in a graded alcohol series, embedded in paraffin, and cut (5 μm sections). Some of these sections were stained with hematoxylin and eosin (H&E); these were used for morphometry and for light microscopical investigation of cytology and architecture of the pulmonary acinus. Other sections were incubated with SAALS and used for type II cell counting. Prior to incubation the sections were deparaffinated in a graded alcohol series. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in phosphate buffered saline (PBS, pH 7.3); to reduce background staining the antibodies were diluted in PBS containing 1% ovalbumine and the sections were incubated with normal goat serum (1:20) for 2h. Then the primary antibody (SAALS 1:50; rabbit anti-mouse) was applied to the sections for overnight incubation, followed by incubation with goat anti-rabbit IgG (1:50; Dakopatts, Denmark) for 1½h, and with rabbit peroxidase anti-peroxidase (PAP 1:500; Nordic Immunological Laboratories, Tilburg, The Netherlands) also for 1½h. Each incubation was followed by rinsing with PBS (3x10 min). Subsequently the sections were stained with 0.04% diamino

benzidine tetrahydrochloride (DAB) in 0.05M tris maleic acid (pH 7.6) with 0.006% H_2O_2 and 0.05% $NiCl_2$ for 10 min. The reaction was stopped with PBS and the sections were counterstained with methyl green.

The fresh-frozen tissue was cut at 6 μm, prefixed by dipping in analytic grade acetone at -20°C (Merck, Darmstadt, FRG) and stored at -20°C. Prior to incubation with antiserum, the sections were further fixed in acetone at -20°C for 10 min and rinsed in PBS. They were incubated with SALS, SALS-E or PS in serial dilutions with PBS (1:10-1:300) for 2 h. After rinsing in PBS they were incubated with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG (Dakopatts, Glostrup, Denmark) for 45 min (indirect immunofluorescence technique). After rinsing in PBS, the sections were mounted under a coverslip in a mixture of 80% glycerol and 20% PBS (pH 8.0) containing p-phenylene diamine (1 mg/ml, which protects slides from fading) and examined with a Leitz Dialux 20 EB immunofluorescence microscope.

Morphometry

Morphometric techniques were used to quantitate morphological differences between control and Nitrofen-exposed lungs on days 19 and 22. For this purpose three rats were selected at random from each experimental group (control group, Nitrofen-exposed group with CDH, and Nitrofen-exposed group without CDH); formalin-fixed, paraffin-embedded lungs were used. Lung volumes were calculated after point counting in 10 to 20 H&E-stained sections, which were chosen at regular intervals through the whole lung, according to Cavalieri's principle (Michel and Cruz-Orive 1988). The same grid was used to determine the volume fractions of future air spaces, lung tissue, and type II cells. Type II cells could be recognized based on immunostaining with SAALS. The data obtained were used to calculate the total volume of lung tissue and the total volume of type II cells. For determination of the surface area of future air spaces a line grid was used (Michel and Cruz-Orive 1988); intercepts of the grid lines with epithelial cells were counted.

Statistics

All statistical comparisons between the three groups were made using a one-way analysis of variance (ANOVA). For the multiple comparison analysis Fisher's LSD method was used. A p value less than or equal to 0.05 was considered indicative of statistical significance. The statistical calculations were done with the statistical package SPSS/PC+.

2.4 Results

Macroscopy

Exposure of Sprague Dawley rats to 100 mg Nitrofen on day 10 of pregnancy resulted in about 75% of the offspring in dorsal diaphragmatic hernias, which were large and left-

sided. In addition, but only in a minority of the cases (23%), a small right-sided dorsal defect was present as well. In the remaining 25% of the offspring Nitrofen-exposure had not resulted in diaphragmatic hernias. In the control group we did not observe any diaphragmatic defects at all. Macroscopically the lung on the side of the (largest) defect was clearly hypoplastic. In younger fetuses and in cases with a small diaphragmatic defect only a small part of the liver was herniated into the thorax. In older fetuses and in cases with larger defects (Fig. 1) a substantial part of the liver, stomach and sometimes bowel loops were present in the thoracic cavity.

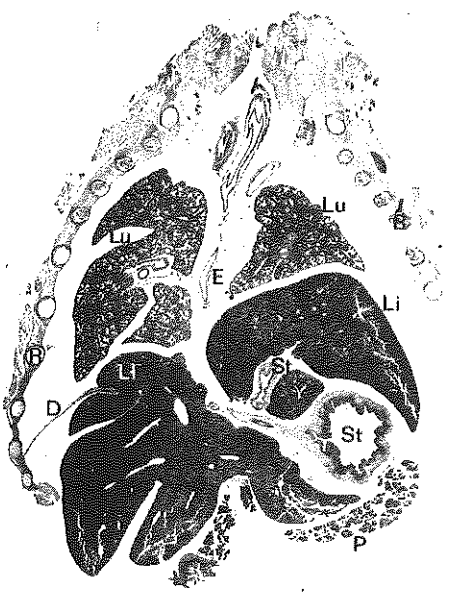

Figure 1 *Frontal section through the dorsal part of the thorax of a 20 day-old-fetal rat, exposed to Nitrofen; formalin-fixation, hematoxylin and eosin (H&E)-staining. This fetus has a large left-sided and a small right-sided diaphragmatic hernia. In this section the left part of the diaphragm is not present at all; liver and stomach are found in the thoracic cavity. The right part of the diaphragm is visible and shows a dorsomedial defect; only a small part of the liver protrudes. (Lu, lung; D, diaphragm; Li, liver; E, esophagus; St, stomach; P, pancreas; R, ribs). 25x.*

Light microscopy

The architecture of the developing lung could be well studied using H&E-stained paraffin sections. These sections showed no difference between control and Nitrofen-exposed lungs on days 18 and 19, irrespective of the presence of a defect in the diaphragm. On these days (Fig. 2) the lungs of all three groups had a pseudoglandular appearance with a respiratory system consisting of tubules lined by approximately cuboid epithelium with large and roundish nuclei, termed acinar tubules (Ten Have-Opbroek 1979; 1991). Acinar tubules are the basic structures for all components of the future pulmonary acinus; they have a round lumen and their lining consists of (immature) type II cells (Ten Have-Opbroek 1981). However, morphometric analysis of the lungs on day 19 showed significantly smaller lung volumes and significantly smaller lung tissue volumes after Nitrofen-exposure, whether or not CDH was present (Table 1). On this day, no other significant differences were found between the control group and the Nitrofen-exposed

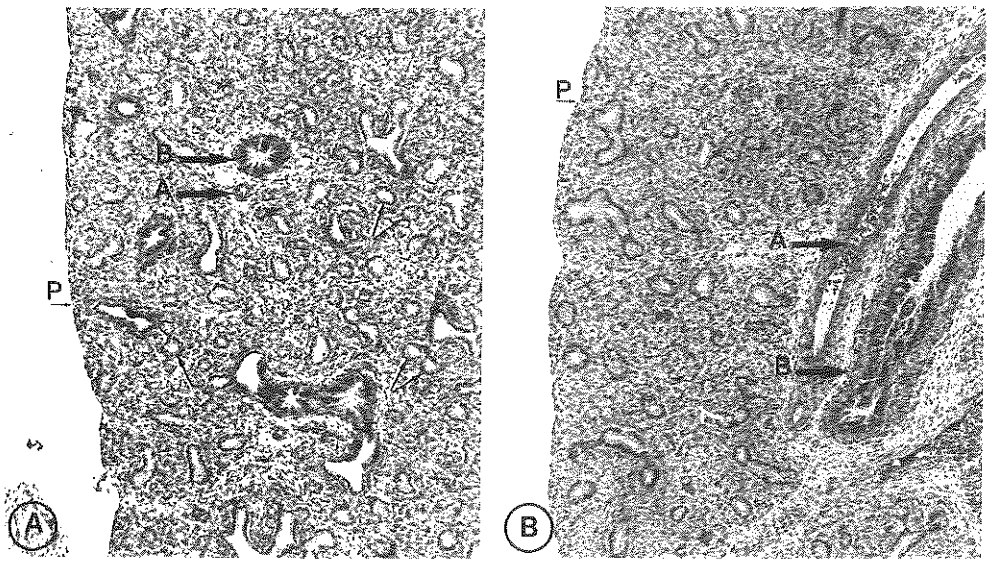

Figure 2 *Fetal rat lungs (day 18) from control and Nitrofen-exposed animals; formalin fixation, H&E staining. (B, bronchiolus; A, pulmonary artery branch; P, pleura).* ***(A)*** *Control group, pseudoglandular period of lung development. The respiratory system consists of acinar tubules only. Acinar tubules (thin arrows), which are the basic components of the future pulmonary acinus, have a small lumen and a lining of cuboid type II cells (see Fig. 7C). 60x.* ***(B)*** *Nitrofen-exposed group with CDH, pseudoglandular period. A similar picture as seen in the control group (see A). 60x.*

groups (Table 1). On day 20 (Figs. 3A,B) all lungs were at the canalicular stage of lung development; the pulmonary acinus now consisted of undilated and more or less dilated acinar tubules. The dilated acinar tubules seemed less numerous in lungs from exposed fetuses with CDH (Fig. 3B) than in those from control fetuses (Fig. 3A) and exposed fetuses without CDH (not shown). The lining of the dilated acinar tubules consisted of cuboid, low cuboid, and thinner cells with an extended basis in both exposed and control lungs. On day 21 (not shown) control lungs and lungs from Nitrofen-exposed fetuses without CDH showed saccular dilations, which means that they had reached the terminal-sac stage of lung development. The saccules were lined by a light microscopically indistinguishable epithelium (composition: cuboid type II cells and more or less thin developing type I cells). Although we also found saccules in lungs from Nitrofen-exposed fetuses with CDH at this age, their incidence seemed lower and their lumens (and those of the dilated acinar tubules) were often slit-like. In addition, septa were thicker and acinar tubules were still numerous. By this, the lungs appeared more compact in Nitrofen-exposed animals with CDH than in those without CDH and in controls. The same picture, but even more pronounced, was found on day 22 (Figs. 4A,B,C). Morphometric analysis

Table 1 Morphometric and statistical analysis of the architecture of fetal rat lungs from the control group, and the Nitrofen-exposed groups (N) with or without congenital diaphragmatic hernia (CDH) on day 19 of gestation (n=3 rats/group).

	Control (1)	N without CDH (2)	N with CDH (3)	Statistical analysis*
Total lung volume	18.2	14.9	14.8	
(mm^3)	16.6	10.8	12.4	
	15.2	13.1	12.8	p=0.06
mean:	16.2	12.9	13.3	1 vs 2,3
% Future air spaces	5.2	4.7	6.1	
	4.7	3.7	6.6	
	5.1	2.0	5.1	p=0.04
mean:	5.0	3.5	5.9	2 vs 3
% Lung tissue	94.8	95.3	93.9	
	95.3	96.3	93.4	
	95.0	98.0	94.9	p=0.04
mean:	95.0	96.5	94.1	2 vs 3
Volume of lung tissue	17.3	14.2	13.9	
(mm^3)	15.8	10.4	11.6	
	14.4	12.8	12.2	p=0.06
mean:	15.8	12.5	12.6	1 vs 2,3
Epithelial surface area	133.7	143.4	169.4	
(mm^2)	167.7	97.9	168.2	
	153.3	113.1	140.5	p=0.08
mean:	151.6	118.1	159.4	2 vs 3

* One-way analysis of variance (ANOVA) was used to compare the three groups and determine the p-value. Fisher's LSD method was used to determine which groups were significantly different from each other.

of the lungs on day 22 showed significant differences between the control group and the Nitrofen-exposed group with CDH and also between the Nitrofen-exposed group without CDH and that with CDH; there were no significant differences between the control group and the Nitrofen-exposed group without CDH. The differences found were smaller lung volumes, smaller epithelial surface areas, smaller volume fractions of future air spaces, and thus larger volume fractions of lung tissue in the Nitrofen-exposed group with CDH. Lung tissue volumes were only significantly smaller in the Nitrofen-exposed group with CDH compared to those in the control group and not compared to those in the Nitrofen-exposed group without CDH (Table 2).

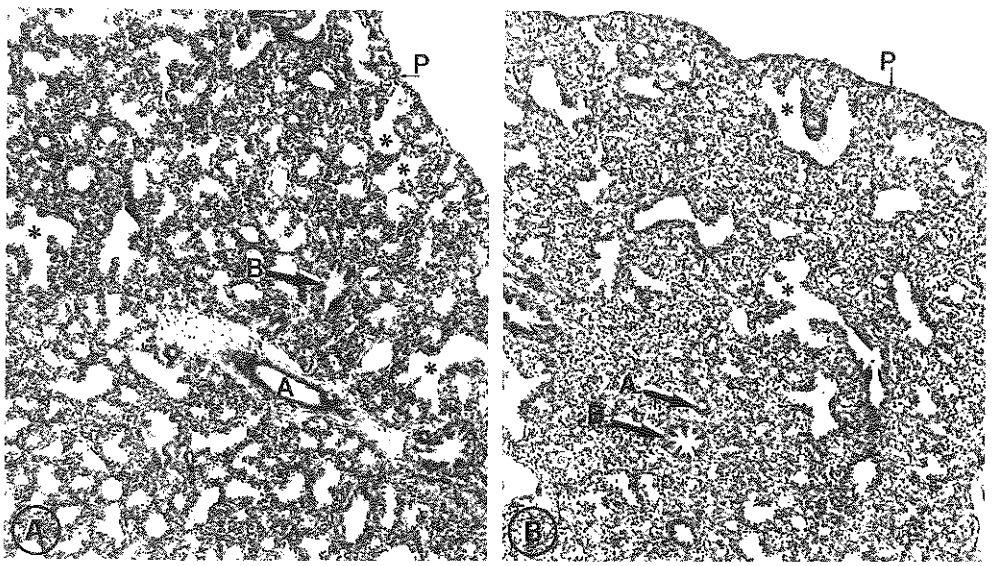

Figure 3 *Fetal rat lungs (day 20) from control and Nitrofen-exposed animals; formalin fixation, H&E staining. (B, bronchiolus; A, pulmonary artery branch; P, pleura).* ***(A)*** *Control group, late canalicular period. There are many dilated acinar tubules (asterisks). 60x.* ***(B)*** *Nitrofen-exposed group with CDH, late canalicular period. The dilated acinar tubules (asterisks) seem fewer in number than in control lungs (Cf. A). 60x.*

All differences between control lungs, and Nitrofen-exposed lungs with and without CDH described above were found in both right and left lungs (Table 2).

Immunohistochemistry

Fluorescence patterns of adult and fetal type II cells: The antiserum SALS (rabbit anti-mouse SP-A) used in the present study in the rat yielded a staining pattern similar to that reported for mouse lungs (Ten Have-Opbroek 1975; 1979; 1981; 1991). In the adult (Fig. 5A) and fetal (Figs. 6,7) rat, type II cells displayed a bright fluorescence of the entire cytoplasm after incubation with SALS and FITC-conjugate. The fluorescent and frequently thick cytoplasmic rim contrasted strongly with the rather dark, large, and roundish nucleus. The fluorescent staining was either diffuse or sometimes more finely or coursely granular. This type of fluorescence is indicated as "cytoplasmic fluorescence". As in other species (Otto-Verberne et al. 1988; Ten Have-Opbroek 1975; 1981; 1991; Ten Have-Opbroek and Plopper 1992), mature type I cells were not immuno reactive. The prospective bronchial epithelium in fetal rat lungs did not display any specific SP-A reactivity; occasionally (Fig. 6A) it showed some staining along the luminal surface. Adult or fetal rat lungs incubated with preimmunization serum, did not display any fluorescence (Fig. 5B), except for an occasional staining along the luminal bronchiolar borders.

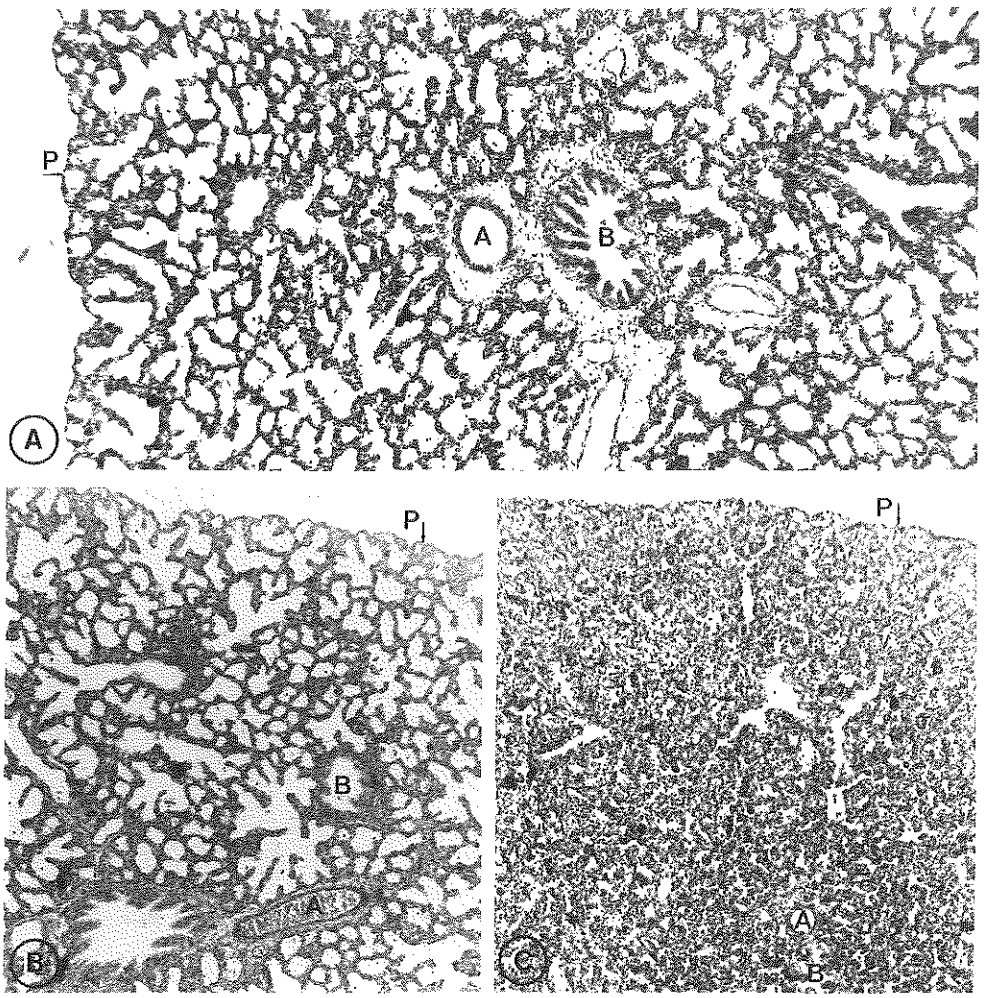

Figure 4 *Fetal rat lungs (day 22) from control and Nitrofen-exposed animals; formalin fixation, H&E staining. (B, bronchiolus; A, pulmonary artery branch; P, pleura).* ***(A)*** *Control group, terminal-sac period. 60x.* ***(B)*** *Nitrofen-exposed group without CDH, normal terminal-sac period (Cf. A). 70x.* ***(C)*** *Nitrofen-exposed group with CDH; the lung has a very compact aspect, which is caused by the presence of fewer saccules, thicker septa, and narrower width of all air-space lumens (Cf. A,B). 60x.*

Fluorescence patterns in normal and Nitrofen-exposed developing fetal rat lungs: On days 18 and 19 (pseudoglandular stage of lung development; not shown) the acinar tubules present were lined by approximately cuboid epithelial cells with large and roundish nuclei and a bright cytoplasmic fluorescence, which was sometimes more striking near the lumen. This fluorescence pattern was seen in lungs from the control group, the Nitrofen-

Table 2 Morphometric and statistical analysis of the architecture of fetal rat lungs from the control group, and the Nitrofen-exposed groups (N) with or without congenital diaphragmatic hernia (CDH) on day 22 of gestation (n=3 rats/group).

	Control (1)			N without CDH (2)			N with CDH (3)		
	left	right	total	left	right	total	left	right	total
Total lung volume (mm^3)	20.7	35.1	55.8	18.0	29.4	47.4	6.3	16.3	22.6
	14.4	30.4	44.8	11.9	23.5	35.4	10.0	18.8	28.9
	21.8	38.0	59.8	16.8	35.6	52.4	9.3	22.0	31.3
	mean:		53.5			45.1			27.6
	p=0.01*								
% Future air spaces	47.0	37.0	42.0	43.2	33.8	38.1	18.3	20.8	20.2
	35.2	37.3	36.4	36.1	35.9	36.0	12.9	15.3	14.3
	39.5	38.9	39.2	43.9	37.8	41.0	16.2	24.1	20.2
	mean:		39.2			38.4			18.2
	p=0.0002*								
% Lung tissue	53.0	63.0	58.0	56.8	66.2	61.9	81.6	79.2	79.8
	64.8	62.7	63.6	63.9	64.1	64.0	87.1	84.7	85.7
	60.5	61.1	60.8	56.1	62.2	59.0	83.8	75.9	79.8
	mean:		60.8			61.6			81.7
	p=0.0002*								
Lung tissue volume (mm^3)	11.0	22.1	32.4	10.2	19.5	29.7	5.1	12.9	18.0
	9.3	19.1	28.5	7.6	15.1	22.7	8.7	15.9	24.8
	13.2	23.2	36.4	9.4	22.2	30.9	7.8	16.7	24.5
	mean:		32.4			27.8			22.4
	p=0.06*								
Epithelial surface area (mm^2)	902.5	1530.4	2432.9	881.5	1211.6	2119.0	226.5	632.2	858.7
	727.2	1295.0	2022.2	584.4	1057.3	1644.1	364.0	757.6	1121.3
	937.4	1653.0	2590.4	698.1	1501.8	2193.2	359.9	902.0	1261.9
	mean:		2348.5			1985.4			1080.6
	p=0.003*								

* One-way analysis of variance (ANOVA) was used to compare the three groups and determine the p-value. Fisher's LSD method was used to determine which groups were significantly different from each other: for all parameters there are significant differences between the three groups; in all cases group 3 differs from groups 1 and 2, except for lung tissue volume where group 3 differs only from group 1.

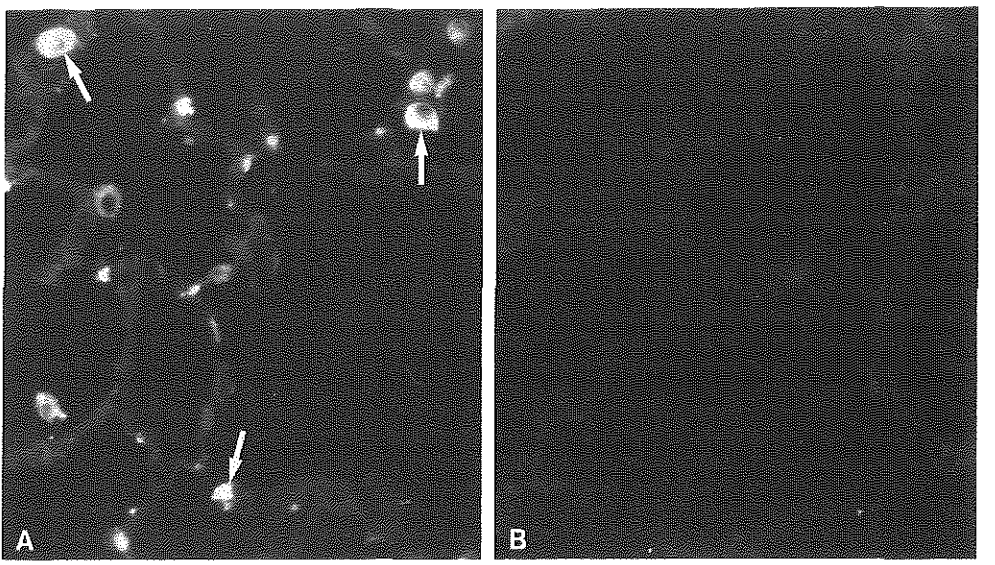

Figure 5 Fresh-frozen sections of rat lung. ***(A)*** *Adult rat lung incubated with SALS (1:300, rabbit anti-mouse SP-A, indirect immunofluorescence). Note the bright cytoplasmic staining of alveolar type II cells (arrows) and the absence of fluorescence where mature type I cells, which are SP-A negative, line the alveolar wall. 350x.* ***(B)*** *Fetal rat lung (day 22) incubated with pre-immunization serum (1:50, indirect immunofluorescence). There is no fluorescence at all. 350x.*

exposed group with CDH, and the Nitrofen-exposed group without CDH. On day 20 (canalicular stage, Fig. 6A,B) in all three groups dilated acinar tubules were present besides undilated forms; these dilated forms were lined by cuboid, low cuboid and thinner cells with an extended basis. These cells all exhibited a cytoplasmic fluorescence pattern, although the intensity of the fluorescence varied considerably. When viewed en face, these thinner cells may be seen as weakly fluorescent sheets dispersed between brightly fluorescent type II cells. On day 21 (early terminal-sac stage; not shown), the epithelial lining of the saccules present consisted of cuboid and thinner cells with fluorescence of the entire cytoplasm and thin cells in which fluorescence was restricted to the borders. In these thin cells, fluorescence was found mainly at the basis of the cell with the nucleus and remaining cytoplasm shadowily visible above it. In control lungs only a linear fluorescence pattern was seen along the luminal surface in some cases and occasionally very thin cells could be found without any fluorescence. The latter two patterns (linear fluorescence and occasional absence of fluorescence) were not observed in Nitrofen-exposed lungs from fetuses with or without CDH. On day 22, in control lungs (Fig. 7A) the saccular lining consisted of some fluorescent cuboid cells interspersed with more or less dark areas, i.e., sites where more or less mature type I cells must occur (see Discussion); truly dark areas (representative for mature type I cells) were also present.

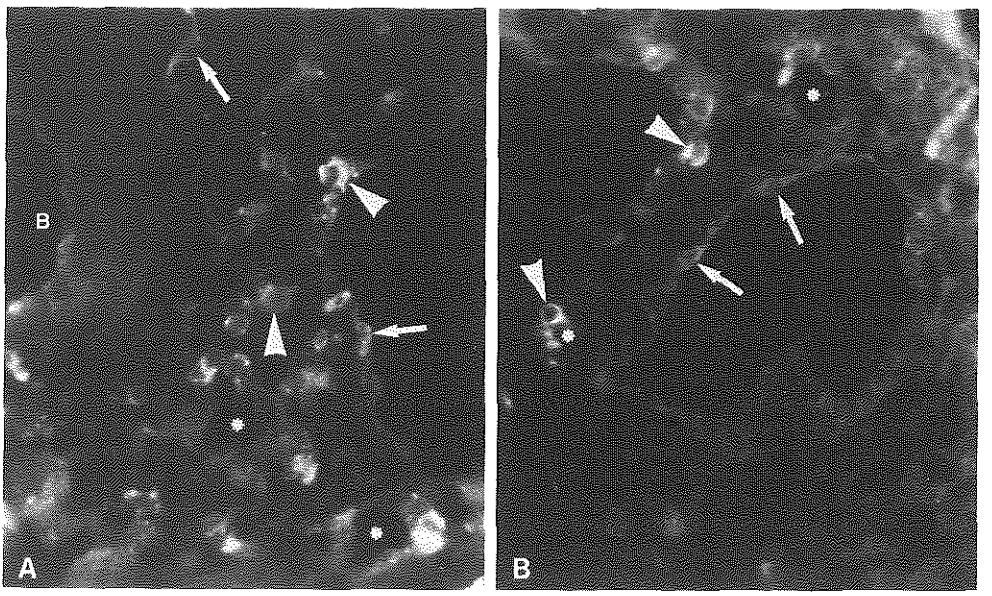

Figure 6 Fetal rat lungs (day 20) from Nitrofen-exposed and unexposed animals; fresh-frozen sections incubated with SALS 1:200 (indirect immunofluorescence). ***(A)*** *Control group; acinar tubules with a small lumen (asterisks) and dilated acinar tubules with approximately cuboid fluorescent type II cells (arrowheads). The dilated tubules also show thinner fluorescent cells with a frequently extended basis (arrows). Bronchial epithelial cells show some fluorescence along the luminal surface, probably caused by presence of surfactant (SP-A) or conjugate between ruffles or cilia (see Discussion). (B, bronchiolus). 350x.* ***(B)*** *Nitrofen-exposed group with CDH; acinar tubules (asterisks) with cuboid fluorescent type II cells (arrowheads) and dilated tubules with also thinner fluorescent cells (arrows); a similar picture as seen in the control group (Cf. A). 350x.*

However, in both groups of Nitrofen-exposed lungs (Fig. 7B,C) the saccules resembled those seen on day 21. They were still lined by thinner (besides cuboid) fluorescent cells and very thin fluorescent cells; few or no dark zones were present in lungs from Nitrofen-exposed fetuses both with (Fig. 7C) and without (Fig. 7B) CDH. Many acinar tubules with cuboid fluorescent epithelium were seen in the lungs from Nitrofen-exposed fetuses

Figure 7 Fetal rat lungs (day 22) from Nitrofen-exposed and unexposed animals; fresh-frozen sections incubated with SALS (indirect immunofluorescence). ***(A)*** *Control group, SALS 1:100; saccules lined with some fluorescent cuboid type II cells (arrowheads) interspersed with more or less dark zones, indicative for the presence of more or less mature type I cells. Note that there are only a few truly dark zones (small arrowheads, Cf. Fig. 5A). 350x.* ***(B)*** *Nitrofen-exposed group without CDH, SALS 1:200; the entire saccular lining is still brightly fluorescent (Cf. A). It shows some fluorescent cuboid type II cells (arrowhead), fluorescent thinner cells (arrows) and linear staining (thin arrows). The latter two patterns suggest retardation of type I cell differentiation in the exposed lungs. 350x.* ***(C)*** *Nitrofen-exposed group with CDH, SALS 1:100; the entire saccular lining is still fluorescent as in lungs from Nitrofen-exposed rats without CDH (Cf. B). In addition these lungs generally show more acinar tubules (asterisks). 350x.*

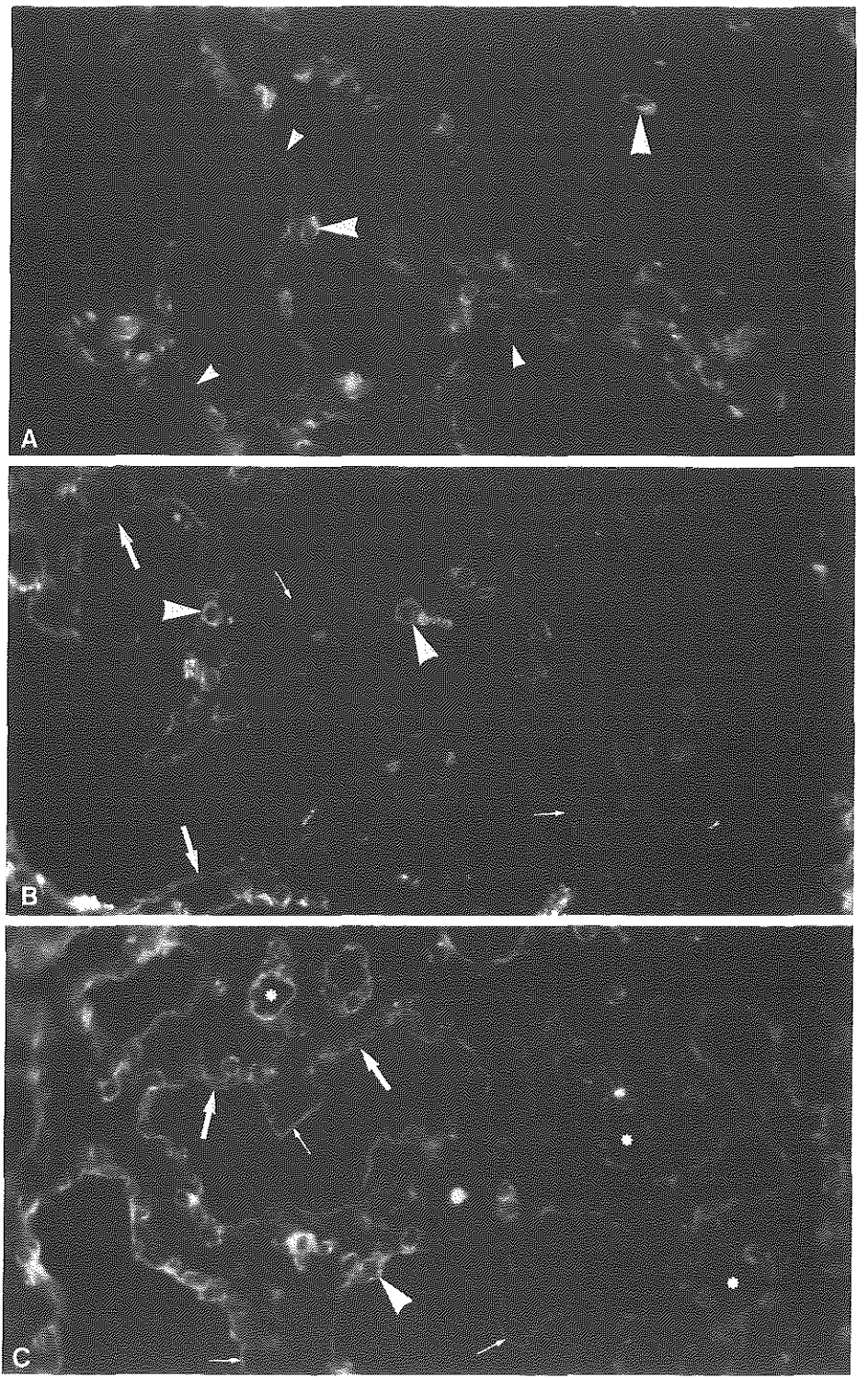
A
B
C

Table 3 Morphometric and statistical analysis of type II cell volumes in fetal rat lungs from the control group, and the Nitrofen-exposed groups (N) with or without congenital diaphragmatic hernia (CDH) on day 19 of gestation (n=3 rats/group).

	Control (1)	N without CDH (2)	N with CDH (3)	Statistical analysis*
% Type II cells in lung tissue	23.9	27.0	24.7	
	24.0	41.2	24.3	
	30.9	23.0	32.6	p=0.73
mean:	26.3	30.4	27.2	-
% Type II cells in total lung	22.5	25.7	23.0	
	22.9	39.7	22.6	
	29.3	22.5	31.1	p=0.68
mean:	24.9	29.3	25.6	-
Total volume of type II cells (mm^3)	4.1	3.8	3.4	
	3.8	4.3	2.8	
	4.5	3.0	4.0	p=0.33
mean:	4.1	3.7	3.4	-

* One-way analysis of variance (ANOVA) was used to compare the three groups and determine the p-value. Fisher's LSD method was used to determine which groups were significantly different from each other.

with CDH. This picture differed greatly from that seen in control lungs (Fig. 7A), where few acinar tubules were seen and relatively many dark zones were present in the saccular lining. The differences between control lungs and Nitrofen-exposed lungs from fetuses with and without CDH described above were found in both the ipsilateral and the contralateral lungs.

Volume fraction of type II cells in normal and Nitrofen-exposed fetal rat lungs: After incubation with SAALS and PAP, followed by staining with DAB and methyl green, type II cells in formalin-fixed paraffin sections displayed a similar cytoplasmic staining pattern as in fresh-frozen sections incubated with SALS and FITC. However, the cytoplasm was now visible as a black rim. Counting the volume fraction of type II cells in total lung tissue revealed no differences between either of the three groups on day 19 (Table 3). On day 22, however, the volume fraction of type II cells in lung tissue was significantly smaller in control lungs than in Nitrofen-exposed lungs without CDH, and also in Nitrofen-exposed lungs with CDH than in those without CDH (Table 4). The difference between control lungs and Nitrofen-exposed lungs with CDH was not significant. The volume fraction of type II cells in total lung was significantly smaller in control lungs than in Nitrofen-exposed lungs without CDH; there were no significant differences between the other groups (Table 4). The total volume of type II cells in the lung was significantly smaller in Nitrofen-exposed lungs with CDH than in control lungs and Nitrofen-exposed lungs without CDH (Table 4).

Table 4 Morphometric and statistical analysis of type II cell volumes in fetal rat lungs from the control group, and the Nitrofen-exposed groups (N) with or without congenital diaphragmatic hernia (CDH) on day 22 of gestation (n=3 rats/group).

	Control (1)	N without CDH (2)	N with CDH (3)	Statistical analysis*
% Type II cells in lung tissue	25.3	29.4	19.1	
	21.2	31.6	20.5	
	26.1	29.7	24.9	p=0.01
mean:	24.2	30.2	21.5	1,3 vs 2
% Type II cells in total lung	14.7	18.4	15.2	
	13.5	20.3	17.6	
	15.9	17.5	19.5	p=0.06
mean:	14.7	18.7	17.4	1 vs 2
Total volume of type II cells (mm^3)	8.2	8.7	3.4	
	6.0	7.2	5.1	
	9.5	9.2	6.1	p=0.04
mean:	7.9	8.4	4.9	3 vs 1,2

* One-way analysis of variance (ANOVA) was used to compare the three groups and determine the p-value. Fisher's LSD method was used to determine which groups were significantly different from each other.

2.5 **Discussion**

SP-A-reactivity and alveolar epithelial cell development in normal fetal lungs

In fetal lungs, identification of specific cell types in the epithelium lining the future air spaces has long been a major problem, because specific cell markers were not available. Light- and electron microscopical studies of mammalian lungs performed in our laboratory have provided detailed information on type II and type I alveolar epithelial cells at early and later stages of prenatal and postnatal lung development (Brandsma et al. 1993; Otto-Verberne et al. 1988; 1990; Otto-Verberne and Ten Have-Opbroek 1987; Ten Have-Opbroek 1975; 1979; 1991; Ten Have-Opbroek and Plopper 1992) and in adult lungs (Ten Have-Opbroek 1986; Ten Have-Opbroek et al. 1991). These studies and other studies of late prenatal, postnatal and adult lungs (Adamson and Bowden 1975; Evan et al. 1975) have shown that type II cells are the stem cells for type I cells during pre- and postnatal lung development. Criteria to recognize mature and immature type II cells at the light microscopical level are the approximately cuboid shape, the large and roundish nucleus, and staining of the entire cytoplasm for the surfactant protein SP-A. In contrast, mature type I cells are squamous, and invisible by light microscopy, and do not show SP-A antigenicity (Otto-Verberne and Ten Have-Opbroek 1987; Otto-Verberne et al. 1988; Ten Have-Opbroek 1975; 1981; 1991; Ten Have-Opbroek and Plopper 1992). Developing type I cells have intermediate cell properties between type II and type I cells (low cuboid to squamous shape; waning cytoplasmic fluorescence) (Otto-Verberne and Ten Have-Opbroek

1987; Otto-Verberne et al. 1988; Ten Have-Opbroek 1975; 1981; 1991; Ten Have-Opbroek and Plopper 1992). In the light of these data, we conclude that the thinner fluorescent cells and the linear fluorescence we found in the pulmonary acinus of fetal rat lungs on days 20-22 after incubation with SALS and FITC-conjugate represent developing type I cells. The fact that developing type I cells are not easily detected in adult lungs may be a result of lower incidence or shorter existence of these transitional forms in mature lungs (Ten Have-Opbroek 1991).

In agreement with earlier findings in prenatal mouse lungs Ten Have-Opbroek 1991) the prospective bronchial epithelium in fetal rat lungs, which consists of columnar ciliated cells and morphologically immature Clara cells, does not display any SP-A reactivity. Occasionally, staining at the luminal surface of a bronchiolus is seen. This occasional fluorescence along the apical border of bronchioles prenatally can also be found in immunohistochemical controls. It must therefore result from retention of conjugate or surfactant (SP-A) between cilia or in epithelial ruffles Ten Have-Opbroek 1991). Cytoplasmic staining prenatally is restricted to type II cells and developing type I cells. Immunoelectron microscopy of prenatal mouse lungs has confirmed all these light microscopical conclusions (Ten Have-Opbroek 1991; Ten Have-Opbroek and de Vries 1993). The same anti-SP-A serum was used to follow the development of the alveolar epithelium in Nitrofen-exposed and control fetal rat lungs.

Effect of Nitrofen-exposure on lung development

As reported by our group (Tenbrinck et al. 1990), exposure of pregnant Sprague Dawley rats to Nitrofen leads to pulmonary hypoplasia in both right and left lungs whether or not CDH is present, shown by individual determination of left and right lung weights, body weight and radial saccular count (RSC). Mean body weights, lung weights, lung weight/body weight ratios, and RSC in the Nitrofen-exposed group with CDH were significantly lower than in the non-exposed control group. No correlation was found between the size of the defect and the ipsilateral lung weight, and, in all cases a significant decrease of lung weight was observed on the contralateral side as well. Right lung weight/left lung weight ratios in the Nitrofen-exposed group did not differ significantly from those in the control group. The Nitrofen-exposed group without CDH showed intermediate values. The results from our present study indicate that this is also true for lung volume and lung tissue volume. A significant decrease of these volumes is observed in the Nitrofen-exposed group with CDH, while right lung/left lung ratios do not differ significantly in either of the groups. In other words, both left and right lungs are affected to the same extent. The exposed group without CDH again showed intermediate values. As further explained below, our study also indicates that hypoplastic fetal rat lungs obtained after Nitrofen-exposure of pregnant Sprague Dawley rats (100 mg on day 10) have a different epithelial composition irrespective of the presence of CDH and a different architecture of the pulmonary acinus only if CDH is present.

Architecture of the pulmonary acinus in hypoplastic lungs

In lungs from control fetuses and Nitrofen-exposed fetuses without CDH, increase of the size of future air spaces and decrease of the thickness of alveolar septa leads to a more open aspect of the lung on day 22 than on day 20. This picture differs greatly from that seen in lungs from Nitrofen-exposed fetuses with CDH: the aspect of the hypoplastic lung is more compact on day 22 than it is on day 20, despite the fact that saccule formation takes place. Larger future air spaces seem less abundant and alveolar septa thicker. This phenomenon becomes more pronounced with increasing gestational age, and is observed in *both* lungs. In other words, there is not a direct correlation between these changes and the site of the diaphragmatic hernia. Morphometric analysis confirms this morphologic observation: the volume fraction of lung tissue is significantly higher in the Nitrofen-exposed group with CDH than in the other two groups. In addition, the ratio of total lung tissue volumes of control lungs and lungs from the Nitrofen-exposed group with CDH is smaller than the ratio of epithelial surface areas of these two groups. This means that in an equal volume of lung tissue a smaller epithelial surface area is present in the Nitrofen-exposed group with CDH, which is in agreement with the morphologic picture of these lungs with less well-developed saccules and thicker septa. Studies in the fetal lamb model in which CDH is created surgically in the second trimester of pregnancy (Pringle et al. 1984; Pringle 1989) also mention an immature appearance of the lung, with smaller air spaces and thicker septa. Ohi et al. (1976) using a similar surgical model in rabbits state that the lungs are not as fully matured as the lungs of normal newborn rabbits, but look more atelectatic than hypoplastic. In their model CDH is created in the third trimester of gestation. The differences between the results from the experiments of Ohi et al. (1976) and Pringle et al. (1984; 1989) might therefore be due to the different timing of CDH creation. In both experiments, however, CDH was created relatively late during gestation, after closure of the pleuroperitoneal canals, while in our rat model the teratologic agent is administered even before the appearance of the lung bud. In the rat model, we are the first to describe the architecture of the lung in detail. It is difficult to compare our results with those in other studies (Kimbrough et al. 1974; Lau et al. 1988; Stone and Manson 1981), because Nitrofen-exposure differs in dose and timing, with divergent results. Lau et al. (1988) find fewer but larger air spaces, while Kimbrough (1974) and Stone and Manson (1981) find atelectasis without any further obvious differences or immaturity. Human case studies of pulmonary hypoplasia associated with CDH (Boyden 1972; George et al. 1987; Nakamura et al. 1991) do mention an immature appearance of the pulmonary acinus but these studies do not further specify the respiratory structures present.

The present study in H&E-stained paraffin sections also demonstrates that in the lungs from Nitrofen-exposed fetuses with CDH the lumens of the air spaces are slit-like and not expanded like those in control lungs and lungs from fetuses without CDH. Similar pictures were shown before in *newborn* rats using about the same rat model (Kimbrough et al. 1974; Stone and Manson 1981), in rabbit lungs (Ohi et al. 1976), and in lungs of term

human patients with CDH (Boyden 1972; Reale and Esterly 1973). Some studies that have used lungs from cases with CDH fixed under pressure after canulation of the trachea, as advocated by Burri et al. (1974) for studying *postnatal* lungs, do not mention the compact picture (George et al. 1987; Kitagawa et al. 1971), which suggests that artificial expansion of the future air spaces took place. However, to the contrary, lungs from our Nitrofen-exposed rats without CDH or from animal models for pulmonary hypoplasia not associated with CDH (Collins et al. 1986; Fisk et al. 1991; Seegmiller et al. 1986) not show this atelectatic picture.

Besides higher volume fractions of lung tissue and smaller total lung volumes, lungs from the Nitrofen-exposed group with CDH also have smaller total lung tissue volumes, which means that these lungs are not only atelectatic, but anyhow smaller. The relatively small epithelial surface areas show that these lungs are also immature. In summary, we find smaller, atelectatic, **and** immature lungs after Nitrofen exposure if CDH is present. Lungs in the Nitrofen-exposed group without CDH are smaller, but not atelectatic and not immature, as shown by normal volume fractions of lung tissue and relatively normal epithelial surface areas. From these findings, we conclude that the presence of viscera in the thoracic cavity protruding through a defect in the diaphragm probably causes the narrow width of the air spaces and thus the compact structure of the lungs. Competition for space in the thorax may also play a role in the abundance of acinar tubules and the deficiency of well developed saccules because this picture is seen in lungs from exposed fetuses with CDH but not in those from fetuses without CDH. Finally, decrease of fetal breathing movements might also contribute to the immaturity of the lungs, as shown in oligohydramnios, chondrodystrophia, or phrenic nerve section (Collins et al. 1986; Fisk et al. 1991; Nagai et al. 1988; Seegmiller et al. 1986). Exposed fetuses without CDH have no impairment of their breathing movements, nor immaturity of the architecture of the lung.

Epithelial composition of the pulmonary acinus in hypoplastic lungs

Comparison of the combined immunohistochemical and H&E-staining results in hypoplastic and control lungs indicates that there are no differences on day 18 and day 19. However, on days 20 through 22 the differences are striking. Based on immunofluorescence pictures, on day 22 the main differences in alveolar epithelial composition between the hypoplastic and control lungs are the following. First, the abundance of mature type I cells in hypoplastic lungs is apparently smaller, suggesting a retarded differentiation of type II into type I alveolar epithelial cells. Second, the abundance of type II cells and developing type I cells seems to be greater in hypoplastic lungs. Morphometric analysis of the lungs shows that volume fractions of type II cells in lung tissue and in total lung are significantly smaller in control lungs than in Nitrofen-exposed lungs without CDH, but not smaller than in those **with** CDH, whereas the total volume of the type II cell population is larger in the control group than in the Nitrofen-

exposed group with CDH. However, when the total epithelial surface area of the lungs of the three groups is taken into account, it turns out that an equally large surface area is occupied by a significantly larger volume of type II cells in the two Nitrofen-exposed groups compared to the control group. Therefore, we can conclude that the alveolar surface present is lined by relatively more type II cells and less type I cells after Nitrofen-treatment. Protruding viscera can not be held responsible for the occurrence of these phenomena, because they occur also in lungs from exposed fetuses without CDH; the pathogenesis is therefore not clear.

Our conclusions regarding the alveolar epithelial maturation in hypoplastic fetal rat lungs are in line with results of other investigators using a similar rat model. Kimbrough et al. (1974) studied the lungs by light- and electron microscopy but only on day 21; they state (based on morphological pictures only) that on that day most alveolar cells are cuboidal and resemble cells of less gestational age. Their conclusion agrees with our finding of the presence of relatively more type II cells around that time. Our conclusions also agree with those obtained in models that interfered later in gestation. In the fetal lamb model, in which pulmonary hypoplasia occurs after surgical creation of a diaphragmatic hernia, increase of the number of mature type II cells is described by several authors (Hashimoto et al. 1985; Pringle et al. 1984). These conclusions however are not based on morphometric data. In a fetal lamb model of pulmonary hypoplasia in association with oligohydramnion, Fisk et al. (1991) have obtained similar results. They find relatively more type II cells and less type I cells in term hypoplastic lungs compared to normal lungs, but they also find many undifferentiated alveolar epithelial cells. They do not describe on which criteria the recognition of these three cell types is based. We are the first to quantitate the differences between normal and hypoplastic lungs using well defined criteria for the recognition of mature and immature type II cells.

These light microscopical and immunofluorescence studies do not allow conclusions about the morphological or functional maturity of the type II cells in hypoplastic lungs in ultrastructural and biochemical respects. Our study describing these aspects has recently been published (Brandsma et al. 1993).

In summary we conclude that Nitrofen-exposed fetal rat lungs: 1) have smaller lung volumes and lung tissue volumes from at least day 19 onwards; 2) are retarded with respect to the differentiation of cuboid type II cells into squamous type I cells; 3) have smaller total volumes of type II cells if CDH is present, but their alveolar surfaces are lined by relatively more type II cells and less type I cells whether or not CDH is present; 4) are atelectatic, and retarded with respect to the development of the future air spaces between days 20 and 22 if CDH is present.

We also conclude that transformation of cuboid fluorescent alveolar type II cells into squamous, not-fluorescent alveolar type I cells starts from day 20 in the fetal rat; the attenuating cells seem to lose their SP-A reactivity first apically and later also basally.

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2.7 References

Adamson IYR, Bowden DH: Derivation of type I epithelium from type II cells in the developing rat lung. Lab Invest 32:736-745, 1975.

Adzick NS, Outwater KM, Harrison MR, Davies P, Glick PL, deLorimier AA, Reid LM: Correction of congenital diaphragmatic hernia in utero IV. An early gestational fetal lamb model for pulmonary vascular morphometric analysis. J Pediatr Surg 20:673-680, 1985.

Areechon W, Reid L: Hypoplasia of the lung with congenital diaphragmatic hernia. Br Med J 1:230-233, 1963.

Askenazi SS, Perlman M: Pulmonary hypoplasia: lung weight and radial alveolar count as criteria of diagnosis. Arch Dis Child 54:614-618, 1979.

Berk C, Grundy M: 'High risk' lecithin/sphingomyelin ratios associated with neonatal diaphragmatic hernia. Case reports. Br J Obstet Gynaecol 89:250-251, 1982.

Boyden EA: The structure of compressed lungs in congenital diaphragmatic hernia. Am J Anat 134:497-507, 1972.

Brandsma AE, Tibboel D, Vulto IM, Egberts J, Ten Have-Opbroek AAW: Ultrastructural features of alveolar epithelial cells in the late fetal pulmonary acinus: a comparison between normal and hypoplastic lungs using a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia. In: Microscopic evaluation of respiratory tract function. Ten Have-Opbroek AAW and Plopper CG, Eds. Microsc Res Techn 26:389-399, 1993.

Burri PH, Dbaly J, Weibel ER: The postnatal growth of the rat lung I. Morphometry. Anat Rec 178:711-730, 1974.

Collins MH, Moessinger AC, Kleinerman J, James LS, Blanc WA: Morphometry of hypoplastic fetal guinea pig lungs following amniotic fluid leak. Pediatr Res 20:955-960, 1986.

Cullen ML, Klein MD, Philippart AI: Congenital diaphragmatic hernia. Surg Clin North Am 65:1115-1138, 1985.

Evans MJ, Cabral LJ, Stephens RJ, Freeman G: Transformation of alveolar type 2 cells to type 1 cells following exposure to NO_2. Exp Mol Pathol 22:142-150, 1975.

Fisk NM, Parkes MJ, Moore PJ, Haidar A, Wigglesworth J, Hanson MA: Fetal Breathing During Chronic Lung Liquid Loss Leading to Pulmonary Hypoplasia. Early Hum Dev 27:53-63, 1991.

Geggel RL, Murphy JD, Langleben D, Crone RK, Vacanti JP, Reid LM: Congenital diaphragmatic hernia: Arterial structural changes and persistent pulmonary hypertension after surgical repair. J Pediatr 107:457-464, 1985.

George DK, Cooney TP, Chiu BK, Thurlbeck WM: Hypoplasia and immaturity of the terminal lung unit (acinus) in congenital diaphragmatic hernia. Am Rev Respir Dis 136:947-950, 1987.

Hashimoto EG, Pringle KC, Soper RT, Brown CK: The creation and repair of diaphragmatic hernia in fetal lambs: morphology of the type II alveolar cell. J Pediatr Surg 20:354-356, 1985.

Harrison MR, Adzick NS, Nakayama DK, deLorimier AA: Fetal diaphragmatic hernia: fetal but fixable. Semin Perinatol 9:103-112, 1985.

Hazebroek FWJ, Tibboel D, Bos AP, Pattenier AW, Madern GC, Bergmeijer JH, Molenaar JC: Congenital diaphragmatic hernia: impact of preoperative stabilization. A prospective pilot study in 13 patients. J Pediatr Surg 23:1139-1146, 1988.

Hisanaga S, Shimokawa H, Kashiwabara Y, Maesato S, Nakano: Unexpectedly low lecithin/sphingomyelin ratio associated with fetal diaphragmatic hernia. Am J Obstet Gynecol 149:905-906, 1984.

Iritani I: Experimental study on embryogenesis of congenital diaphragmatic hernia. Anat Embryol 169:133-139, 1984.

Kimbrough RD, Gaines TB, Linder RE: 2,4-Dichlorophenyl-p-nitrophenyl ether (TOK): effects on the lung maturation of rat fetus. Arch Environ Health 28:316-320, 1974.

Kitagawa M, Hislop A, Boyden EA, Reid L: Lung hypoplasia in congenital diaphragmatic hernia. A quantitative study of airway, artery, and alveolar development. Br J Surg 58:342-346, 1971.

Kluth D, Kangah R, Reich P, Tenbrinck R, Tibboel D, Lambrecht W: Nitrofen-induced diaphragmatic hernias in rats: an animal model. J Pediatr Surg 25:850-854, 1990.

Kluth D, Tenbrinck R, von Ekesparre M, Kangah R, Reich P, Brandsma A, Tibboel D, Lambrecht W: The natural history of congenital diaphragmatic hernia and pulmonary hypoplasia in the embryo. J Pediatr Surg 28:456-463, 1993.

Lau C, Cameron AM, Irsula O, Antolick LL, Langston C, Kavlock RJ: Teratogenic effects of nitrofen on cellular and functional maturation of the rat lung. Toxicol Appl Pharmacol 95:412-422, 1988.

Michel RP, Cruz-Orive LM: Application of the Cavalieri principle and vertical sections method to lung: estimation of volume and pleural surface area. J Microsc 150:117-136, 1988.

Molenaar JC, Bos AP, Hazebroek FWJ, Tibboel D: Congenital diaphragmatic hernia, what defect? J Pediatr Surg 26:248-254, 1991.

Nagai A, Thurlbeck WM, Jansen AH, Ioffe S, Chernick V: The effect of chronic biphrenectomy on lung growth and maturation in fetal lambs. Morphologic and morphometric studies. Am Rev Respir Dis 137:167-172, 1988.

Nakamura Y, Yamamoto I, Fukuda S, Hashimoto T: Pulmonary acinar development in diaphragmatic hernia. Arch Pathol Lab Med 115:372-376, 1991.

Nakao Y, Ueki R, Nakao Y, Nishida T, Nomura M, Takahashi T, Nakao BMS: Effects of maternal nitrofen exposure on lung organogenesis in mice and rats. Teratology 43A, 1990.

Ohi R, Suzuki H, Kato T, Kasai M: Development of the lung in fetal rabbits with experimental diaphragmatic hernia. J Pediatr Surg 11:955-959, 1976.

Oomen LCJ, Ten Have-Opbroek AAW, Hageman PC, Oudshoorn-Snoek M, Egberts J, van der Valk MA, Calafat J, Demant P: Fetal mouse alveolar type II cells in culture express several type II cell characteristics found in vivo, together with major histocompatibility antigens. Am J Respir Cell Mol Biol 3:325-339, 1990.

Otto-Verberne CJM, Ten Have-Opbroek AAW: Development of the pulmonary acinus in fetal rat lung: a study based on an antiserum recognizing surfactant-associated proteins. Anat Embryol 175:365-373, 1987.

Otto-Verberne CJM, Ten Have-Opbroek AAW, Balkema JJ, Franken C: Detection of the type II cell or its precursor before week 20 of human gestation, using antibodies against surfactant-associated proteins. Anat Embryol 178:29-39, 1988.

Otto-Verberne CJM, Ten Have-Opbroek AAW, De Vries ECP: Expression of the major surfactant-associated protein, SP-A, in type II cells of human lung before 20 weeks of gestation. Eur J Cell Biol 53:13-19, 1990.

Pringle KC, Turner JW, Schofield JC, Soper RT: Creation and repair of diaphragmatic hernia in the fetal lamb: lung development and morphology. J Pediatr Surg 19:131-140, 1984.

Pringle KC: Lung development in congenital diaphragmatic hernia. In: Puri P, ed., Congenital Diaphragmatic Hernia. Mod Probl Paediatr, Basel, Karger 24:28-53, 1989.

Puri P, Dewan P: Historical review. In: Puri P, ed., Congenital Diaphragmatic Hernia. Mod Probl Paediatr, Basel, Karger 24:1-6, 1989.

Reale FR, Esterly JR: Pulmonary hypoplasia: a morphometric study of the lungs of infants with diaphragmatic hernia, anencephaly, and renal malformations. Pediatrics 51:91-96, 1973.

Seegmiller RE, Cooper CA, Houghton MJ, Carey JC: Pulmonary hypoplasia in chondrodystrophic mice. Teratology 33:339-347, 1986.

Stone LC, Manson JM: Effects of the herbicide 2,4-dichlorophenyl-p-nitrophenyl ether (nitrofen) on fetal lung development in rats. Toxicology 20:195-207, 1981.

Tenbrinck R, Tibboel D, Gaillard JLJ, Kluth D, Bos AP, Lachmann B, Molenaar JC: Experimentally induced congenital diaphragmatic hernia in rats. J Pediatr Surg 25:426-429, 1990.

Ten Have-Opbroek AAW: Immunological study of lung development in the mouse embryo. Appearance of a lung-specific antigen, localized in the great alveolar cell. Dev Biol 46:390-403, 1975.

Ten Have-Opbroek AAW: Immunological study of lung development in the mouse embryo. II. First appearance of the great alveolar cell, as shown by immunofluorescence microscopy. Dev Biol 69:408-423, 1979.

Ten Have-Opbroek AAW: The development of the lung in mammals: an analysis of concepts and findings. Am J Anat 162:201-219, 1981.

Ten Have-Opbroek AAW: The structural composition of the pulmonary acinus in the mouse. A scanning electron microscopical and developmental-biological analysis. Anat Embryol 174:49-57, 1986.

Ten Have-Opbroek AAW: Lung development in the mouse embryo. Exp Lung Res 17:111-130, 1991.

Ten Have-Opbroek AAW, Otto-Verberne CJM, Dubbeldam JA, Dykman JH: The proximal border of the human respiratory unit, as shown by scanning and transmission electron microscopy and light microscopical cytochemistry. Anat Rec 229:339-354, 1991.

Ten Have-Opbroek AAW, Plopper CG: Morphogenetic and Functional Activity of Type II Cells in Early Fetal Rhesus Monkey Lungs - A Comparison Between Primates and Rodents. Anat Rec 234:93-104, 1992.

Ten Have-Opbroek AAW, De Vries ECP: Clara cell differentiation in the mouse: ultrastructural morphology and cytochemistry for Surfactant Protein A and Clara cell 10 kD protein. In: Microscopic evaluation of respiratory tract function. Ten Have-Opbroek AAW and Plopper CG, Eds. Microsc Res Techn 26:400-411, 1993.

Tibboel D, Tenbrinck R, Brandsma AE, Bos AP, Sluiter W, Kluth D, Ten Have-Opbroek AAW, Lachmann B, Molenaar JC: Pathogenetic and functional aspects of CDH: an experimental study. In: Kachel W, Wischnik A, Melchert F, Niessen KH, Eds., Interdisziplinäre Probleme in der Perinatalmedizin. Braun G, Fachverlage, Karlsruhe, 8-11, 1993.

Ueki R, Nakao Y, Nishida T, Nakao Y, Wakabayashi T: Lung hypoplasia in developing mice and rats induced by maternal exposure to Nitrofen. Cong Anom 30:133-143, 1990.

Van Hemert FJ, Ten Have-Opbroek AAW, Otto-Verberne CJM: Histochemical characterization of an antigen specific for the great alveolar cell in the mouse lung. Histochemistry 85:497-504, 1986.

Wenstrom KD, Weiner CP, Hanson JW: A Five-Year Statewide Experience with Congenital Diaphragmatic Hernia. Am J Obstet Gynecol 165:838-842, 1991.

Chapter 3

ULTRASTRUCTURAL FEATURES OF ALVEOLAR EPITHELIAL CELLS IN THE LATE FETAL PULMONARY ACINUS

A comparison between normal and hypoplastic lungs, using a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia

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ULTRASTRUCTURAL FEATURES OF ALVEOLAR EPITHELIAL CELLS IN THE LATE FETAL PULMONARY ACINUS

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3.1 Abstract

Aim of this study was to describe and compare the ultrastructural features and functional maturity of alveolar epithelial cells in hypoplastic and normal fetal rat lungs. Pulmonary hypoplasia in association with congenital diaphragmatic hernia was induced in fetuses by administration of 2,4-dichlorophenyl-p-nitrophenylether (Nitrofen) to pregnant Sprague Dawley rats (100 mg on day 10 of gestation). Lung tissue of Nitrofen-exposed and control fetal rats aged 19-22 days (vaginal plug day 1, birth day 23) was embedded in Epon. Semithin (1 μm) toluidine blue-stained sections were examined by light microscopy; ultrathin sections (ca.80 nm) were studied by transmission electron microscopy. In bronchoalveolar lavage fluid from control and Nitrofen-exposed fetuses (day 22) phospholipid fractions and surfactant protein A content were measured (semi) quantitatively. On day 19 both control and Nitrofen-exposed lungs contained only cuboid alveolar epithelial cells; from day 20 there were cuboid, low cuboid and thinner epithelial cells. The (low) cuboid cells contained large glycogen fields, some precursory stages of multilamellar bodies (MLBs), and just a few mature MLBs on day 19 and 20; smaller glycogen fields, more precursory stages and more mature MLBs on day 21; and little or no glycogen but many precursory stages and mature MLBs on day 22. The thinner cells contained little or no glycogen and a few precursory stages of MLBs on days 20 through 22; very thin cells on day 22 contained neither glycogen nor any precursory stages of MLBs. MLBs and tubular myelin were seen in the lumens of future air spaces from day 20 onward. Nitrofen-exposed lungs differed from control lungs in that inclusion bodies (IBs) were less numerous in (low) cuboid alveolar cells on days 19 and 20, and more glycogen was seen on day 22. In addition, intra- and extracellular "MLBs" in exposed lungs more often had an unusual appearance, i.e. a confluent structure and higher electron density. However, despite morphologic differences, there was no clear difference in phospholipid composition and SP-A-content per mol phospholipid in bronchoalveolar lavage fluid.

We concluded that morphologically hypoplastic lungs are less mature near term, without an apparent effect on surfactant composition.

3.2 **Introduction**

Congenital diaphragmatic hernia (CDH) is a serious anomaly in humans. The incidence of CDH is 1:3000 newborns, the etiology is unknown and the mortality rate is 40-60% (Wenstrom et al. 1991). One of the main causes of death in children with CDH is the associated pulmonary hypoplasia (review Molenaar et al. 1991). Improvement of therapeutic possibilities for children with respiratory distress due to pulmonary hypoplasia requires insight in normal and abnormal pulmonary development, morphology and biochemistry. Studies in human cases hardly allow these kinds of investigations at specific prenatal time-points, or without any additional damage by mechanical ventilation (barotrauma, oxygen toxicity). Therefore, to obtain further information, we use a rat model (Kluth et al. 1990; Tenbrinck et al. 1990), in which pulmonary hypoplasia and CDH are induced by the herbicide 2,4-dichlorophenyl-p-nitrophenylether (Nitrofen) administered to the mother on day 10 of pregnancy. Administration of a single dose of this teratologic agent very early in gestation, i.e. 6 days before normal closure of the pleuroperitoneal canals and just before anlage of the lung primordium, interferes with diaphragmatic and pulmonary development. Nitrofen-exposed neonatal rat lungs are hypoplastic on both left and right side (Lau et al. 1988; Stone and Manson 1981; Tenbrinck et al. 1990) according to the criteria of Askenazi and Perlman (1979) for human species. Macroscopically, this exposure results in dorsal diaphragmatic hernias, which are large and left-sided, in about 80% of the offspring. In addition, but only in a minority of the cases (26%), a small right-sided dorsal defect is present as well. In younger fetuses and in cases with a small diaphragmatic defect only a small part of the liver herniates into the thorax, whereas in older fetuses and in cases with larger defects a substantial part of the liver, stomach, and sometimes bowel loops are present in the thorax as well. In Nitrofen-exposed fetuses without CDH the lungs are also hypoplastic (Tenbrinck et al. 1990). Nitrofen possibly inhibits early lung proliferation or differentiation via alterations in the thyroid hormone status of the embryo (Manson 1986) by a direct interaction with the nuclear thyroid hormone receptor (our own unpublished results). The existence of such an early effect of Nitrofen on lung development would explain why results from studies in which CDH is created surgically in fetal lambs, differ from those obtained in our study (see discussion). Light microscopically, there is a retarded differentiation of cuboid type II cells into squamous type I cells and a retarded development of the future air spaces between days 20 and 22 in lungs from Nitrofen-exposed fetuses with CDH (Brandsma et al. 1992a). However, the techniques used (light microscopy and indirect immunofluorescence) do not allow conclusions about the morphological or functional maturity of the alveolar epithelial cells.

Therefore, aim of the present study was to investigate the ultrastructural morphology and functional maturity of type I and type II cells in the developing hypoplastic fetal rat lung. For general orientation semithin Epon sections were stained with toluidine blue and

examined by light microscopy. To study alveolar epithelial cells ultrathin Epon sections were examined by transmission electron microscopy (TEM). Morphological and functional maturity of type II cells was determined by TEM (presence of precursory forms or mature multilamellar bodies; Kikkawa and Spitzer 1969; Ten Have-Opbroek et al. 1988, 1990) and by (semi)quantitative measurement of phospholipid- and SP-A-content in bronchoalveolar lavage fluid on day 22. The results were compared with our findings in normal fetal rat lungs.

3.3 **Materials and Methods**

Animal treatment

Pulmonary hypoplasia in association with CDH was induced as described before (Kluth et al. 1990; Tenbrinck et al. 1990): Adult Sprague Dawley rats were mated overnight. The next day, if a vaginal plug was present, was considered to be day one of pregnancy. A single dose of 100 mg Nitrofen dissolved in olive-oil was administered to the ether-drowsed mothers through an intragastric tube on day 10 of pregnancy. 85 Nitrofen-exposed fetuses and 50 control fetuses were collected by Ceasarian section from day 18 through day 22 (birth on day 23). They were weighed and pithed. A number of control and Nitrofen-exposed fetuses underwent bronchoalveolar lavage. Finally, in all fetuses presence, position, and size of a diaphragmatic defect were evaluated.

Microscopy

From 19 Nitrofen-exposed fetuses with CDH and 17 age-matched control fetuses (day 18-22) the lungs were dissected out and fixed by immersion in 2% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 0.075% $CaCl_2$ for 24 hr at 4°C. The tissue was postfixed for 2 hr in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) at room temperature. After dehydration via a graded alcohol series (70-100%, each step lasting 15 min), the tissue was transferred to a mixture of propylene oxide and Epon (1:1) for 45 min, stored overnight in Epon in an exsiccator under low vacuum for better penetration of Epon into the lung tissue, and then embedded in fresh Epon.

Semithin sections (1 μm) were cut, using a glass knife and a LKB ultramicrotome (LKB-produkter AB, Stockholm, Sweden). The sections were stained with toluidine blue and used for light microscopical investigation of cytology and architecture of the pulmonary acinus and for selection of representative fields for electron microscopy. Ultrathin sections (ca. 80 nm) were cut with a LKB ultramicrotome using a diamond knife (Diatome Ltd., Bienne, Switzerland). The sections were mounted on copper R100a grids (Veco, Eerbeek, The Netherlands), stained with uranyl acetate 7% for 45 min and lead citrate for 10 min, and examined with a Philips 201 electron microscope at 60 kV.

Bronchoalveolar lavage

33 Control and 52 Nitrofen-exposed fetuses with CDH (day 22) underwent bronchoalveolar lavage. After inserting a tube into the trachea, the lungs were washed in situ with 0.05-0.35 ml 0.9% NaCl (37°C) depending on air space volume, 3-10 times or until leakage occurred. Each lavage volume was twice infused and withdrawn slowly. To preserve surfactant associated proteins the protease-inhibitor phenylmethylsulfonyl fluoride (PMSF, 0,25 mM) was added to the lavage fluid. Then the lavage fluid was centrifuged for 10 min at 350 g to remove cells and macrophages, saturated with nitrogen to prevent degradation, and stored at -20°C. If required, lavage fluid from a number of animals was pooled to get appropriate volumes for further processing.

Phospholipids in bronchoalveolar lavage fluid

Lipids were extracted from the lavage fluid with dichloromethane and methanol (2:1 v/v). Total phospholipid content was determined as described before (Fiske and Subbarow 1925). The various phospholipid classes were separated by thin-layer chromatography on HPTLC plates (Merck, 60F254, Germany), using chloroform / methanol / 2-propanol / 0.25% KCl / triethylamine (30:9:25:6:18, v/v/v/v/v) as mobile phase (Touchstone et al. 1980). The plates were dried and the spots visualized under UV light (254 nm) after spraying the plates with 0.01% Rhodamine 6G in distilled water. The spots were scraped off the plates and their percentages were calculated on the basis of phosphorus determinations.

Surfactant Protein A in bronchoalveolar lavage fluid

To compare SP-A contents in lavage fluid from fetal Nitrofen-exposed and control rats a dot blot assay was performed. For this purpose lavage fluid was centrifuged for 1 hr at 20,000g. The pellets were resuspended in 0.9% NaCl; the final volumes were adjusted with 0.9% NaCl to obtain equal concentrations of phospholipid in lavage material from Nitrofen-exposed and control rats. Drops with predetermined concentrations of phospholipid were spotted on nitrocellulose paper. After drying, the blots were incubated for three hours with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) and subsequently incubated overnight with our anti-SP-A antiserum (rabbit anti-mouse; 1:400; Oomen et al., 1990) or with pre-immunization serum as a negative control. After rinsing 3x20 min with PBS the blots were incubated with SWAR-peroxidase (swine anti-rabbit) conjugate for 90 min, rinsed again with PBS (3x20 min) and stained with diamino benzidine (DAB) in TRIS buffer (pH 7.4) for 1-15 min. The staining was stopped with 0.4% ß-mercapto-ethanol as soon as the staining visually was considered to be optimal. Finally the blots were rinsed with distilled water and dried overnight on filtration paper. Bronchoalveolar lavage fluid from patients with alveolar proteinosis was used as a positive control, and normal human, mouse, and rat serum as negative controls.

3.4 Results

Light microscopy

In general, lung development in Nitrofen-exposed and control rats followed the pathway proposed for mammals (Fig. 1). On day 19 (not shown), control and exposed lungs looked similar and were at the pseudoglandular stage of lung development. The prospective pulmonary acinus consisted of tubules with small round lumens, lined by approximately cuboid epithelial cells with large and roundish nuclei (acinar tubules with cuboid type II cells, see Fig. 1B). Differences between Nitrofen-exposed and control lungs were present from day 20 onward and became more pronounced with time. On day 20 (canalicular stage, not illustrated), some tubules were slightly dilated and lined by cuboid and thinner cells; these tubules were less numerous in exposed lungs. On days 21 and 22, control and exposed lungs were in the saccular stage of lung development (Fig. 2A,B). The saccular lining consisted of some (low) cuboid cells interspersed with a few (day 21) or many (day 22) thin cells in control lungs, and cuboid and low cuboid to thin cells in exposed lungs. In the exposed lungs, the lumens of air spaces were frequently slit-like, and acinar tubules were more abundant than in control lungs. These phenomena gave the exposed lungs a primitive, compact aspect compared to control lungs (Fig. 2). For further details, see Brandsma et al. (1992a).

Transmission Electron Microscopy

On day 19 the acinar tubules of the respiratory system in control (Fig. 3A) and Nitrofen-exposed (not shown) fetal rats were lined exclusively by low columnar or cuboid epithelial cells with large and roundish nuclei. These approximately cuboid cells contained large glycogen fields and a few inclusion bodies (IBs; Figs. 3B-D). Most of the IBs were cytoplasmic IBs (Fig. 3B) situated in or near a glycogen field. Cytoplasmic IBs usually contain glycogen particles, and are bounded by one or more membranes of smooth endoplasmic reticulum (ER); they probably are primary stages in prenatal multilamellar body (MLB) formation (Ten Have-Opbroek et al. 1990). In addition some dense bodies (DBs; Fig. 3B), osmiophilic multivesicular bodies (MVBs; Fig. 3C) and occasionally IBs with a few lamellae, so-called lamellar bodies (LBs; Ten Have-Opbroek 1990) were present (Fig. 3D). In exposed lungs (Fig. 3E) the findings were similar, except that some LBs had a higher electron density and confluent thick lamellae.

In both groups the dilated acinar tubules on day 20 (Fig. 4A) were lined by cuboid cells, low cuboid cells with an extended basis, and some thinner cells. Cuboid and low cuboid cells still contained large glycogen fields, and the same types of IBs as on day 19, but more abundantly. In addition, mature MLBs were seen in cuboid cells. The thinner cells contained little or no glycogen and a few IBs; most of these IBs were cytoplasmic IBs, but DBs (Fig. 4B) and osmiophilic MVBs (Fig. 4C) were also seen. The future air spaces contained a few MLBs and some tubular myelin. In Nitrofen-exposed lungs, again, a

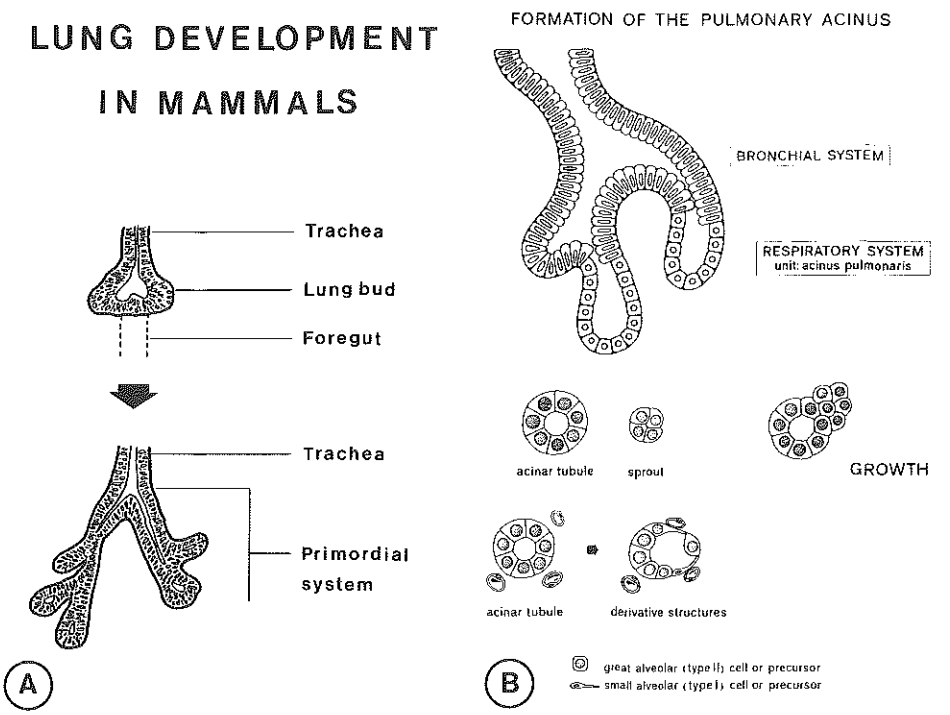


Figure 1 Diagrams of mammalian lung development (Ten Have-Opbroek, 1981) (A) The two lung buds arising from the primitive foregut (in the rat by day 11) develop into the primordial systems of the right and left lungs. The undifferentiated epithelium lining the primordial tubules is pseudostratified columnar. (B) The prospective bronchial system and the prospective respiratory system (unit: pulmonary acinus) differentiate from the primordial system, in the rat by day 16. The basic structure in pulmonary acinus formation is the acinar tubule or sprout (lining: type II cells). Transformation of acinar tubules results in derivative structures (dilated tubules, saccules, alveoli), which contain not only cuboid type II cells in their lining, but also thinner (developing) type I cells (in the rat as of day 20).

similar picture was seen but MLBs in (low) cuboid cells were not as abundant as in control lungs.

On day 21 the lining of the saccules in lungs from both groups consisted of some cuboid and low cuboid cells and many thinner epithelial cells. In the cuboid cells approximately half of the IBs were estimated to be cytoplasmic IBs in both exposed and control lungs, the other half consisted of DBs, MVBs and MLBs. Many cuboid cells as well as low cuboid cells contained large glycogen fields. In many low cuboid cells MLBs were seen. The thinner epithelial cells frequently contained little glycogen and a single DB, but in some of these cells osmiophilic MVBs, cytoplasmatic IBs, or LBs (Fig. 4D) were present. The future air spaces of both Nitrofen-exposed and control lungs contained tubular myelin and MLBs.

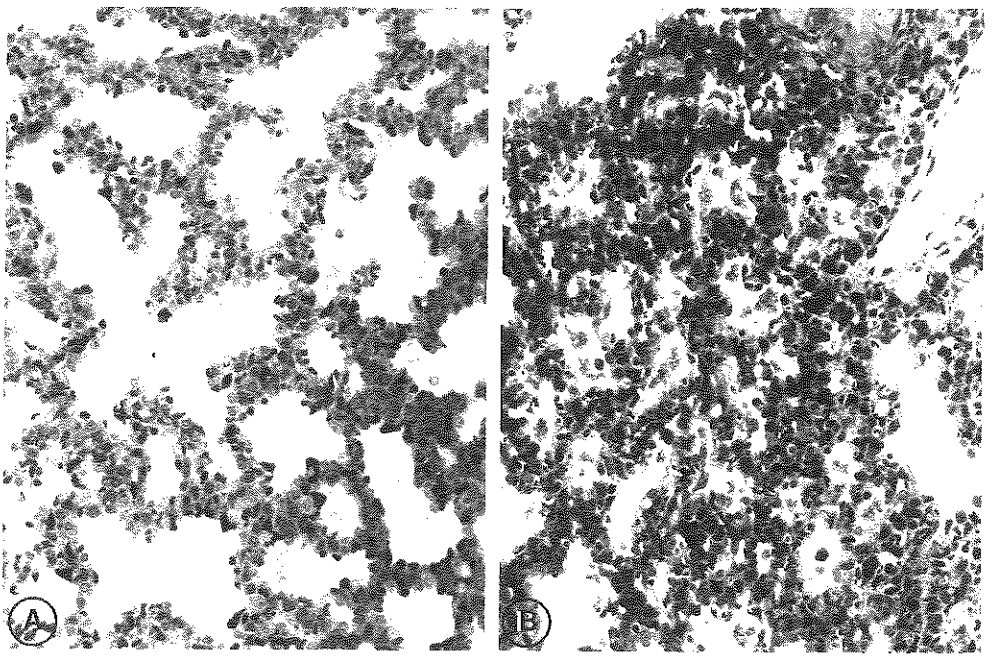
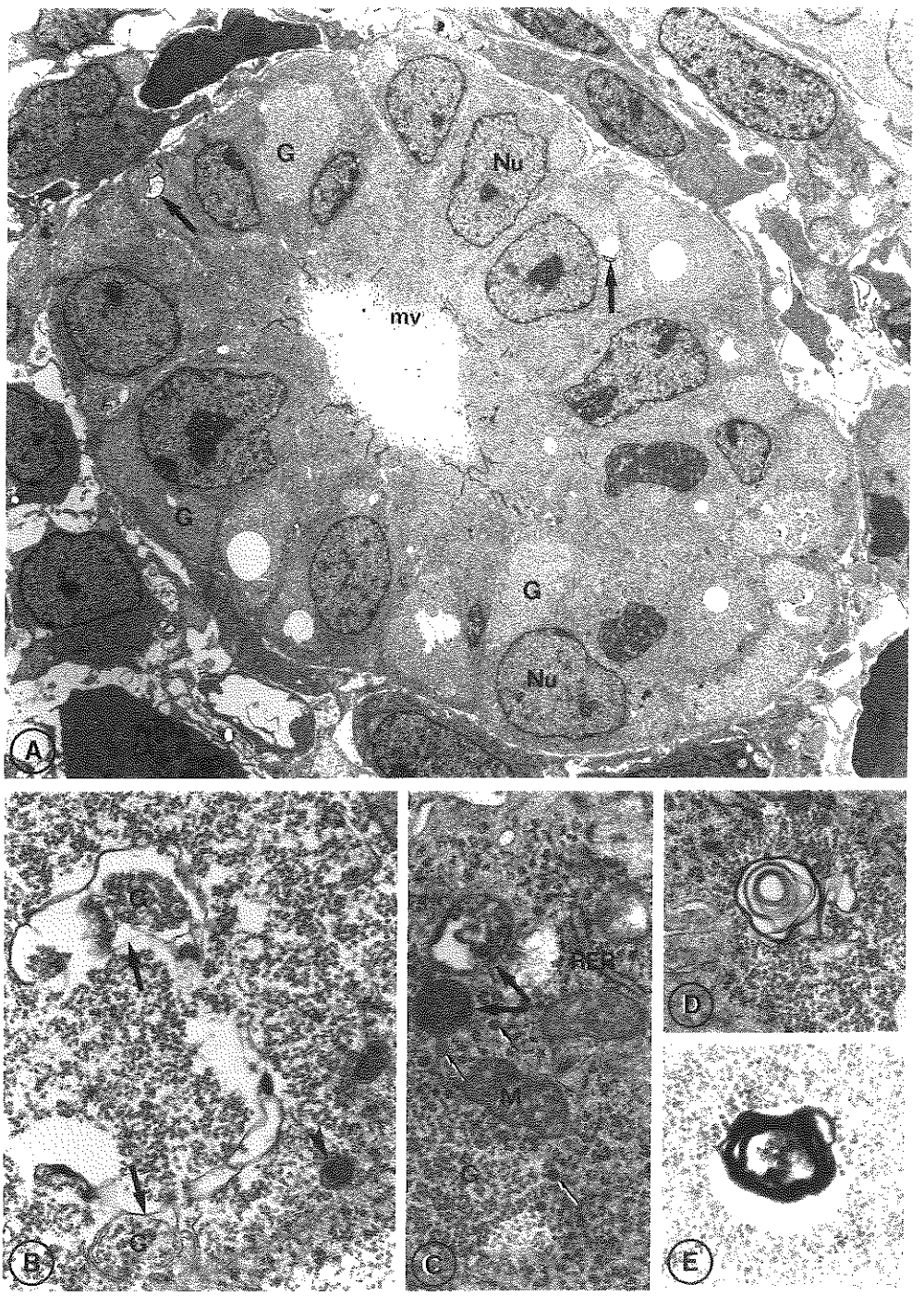


Figure 2 Fetal rat lungs, day 22; 1 μm Epon sections, Toluidine blue staining, 225x. (A) Control group, normal terminal sac stage. (B) Nitrofen-exposed group, the lung has a very compact aspect.

On day 22 (Fig. 5) many thin epithelial cells and some cuboid cells lined the saccules. In control lungs most cuboid cells (Fig. 5A) contained little or no glycogen, but some still had large glycogen fields. Many IBs were present; after random counting we estimated that about half of the IBs were MLBs, and the other half cytoplasmic IBs, MVBs and DBs. In cuboid cells in exposed lungs (Fig. 5B), generally more glycogen was seen. The thin epithelial cells in control and Nitrofen-exposed lungs were like those on day 21, but contained less IBs. MLBs and tubular myelin were present in the future air spaces (Fig. 5C,D).

Figure 3 Transmission electron microscopical (TEM) pictures of developing type II cells in control (a-d) and Nitrofen-exposed (e) fetal rat lungs; day 19. (Nu, nucleus; G, glycogen; M, mitochondrion; RER, rough endoplasmic reticulum). (A) Acinar tubule lined by approximately cuboid (type II) cells with large and roundish nuclei and microvilli (mv). Note the large apical or basal glycogen fields and the presence of some electron dense inclusion bodies (IBs; arrows). For details, see b-d. 2.240x. (B) Cytoplasmic IBs (arrows), containing glycogen and surrounded by smooth membranes; these IBs presumably are primary stages in MLB formation. Glycogen is easily lost during tissue processing. A dense body (arrowhead), probably representing another stage in MLB formation, is also present. 19.000x. (C) Osmiophilic MVBs (arrows), also postulated precursors in MLB formation, and some discrete (un)coated vesicles (thin arrows) in the proximity of MVBs and glycogen. 19.000x. (D) Lamellar body surrounded by glycogen. 19.000x. (E) Lamellar body in a large glycogen field in a type II cell of a Nitrofen-exposed fetus. Note the high density and confluent thick lamellae (Cf. d.). 26.000x.


G
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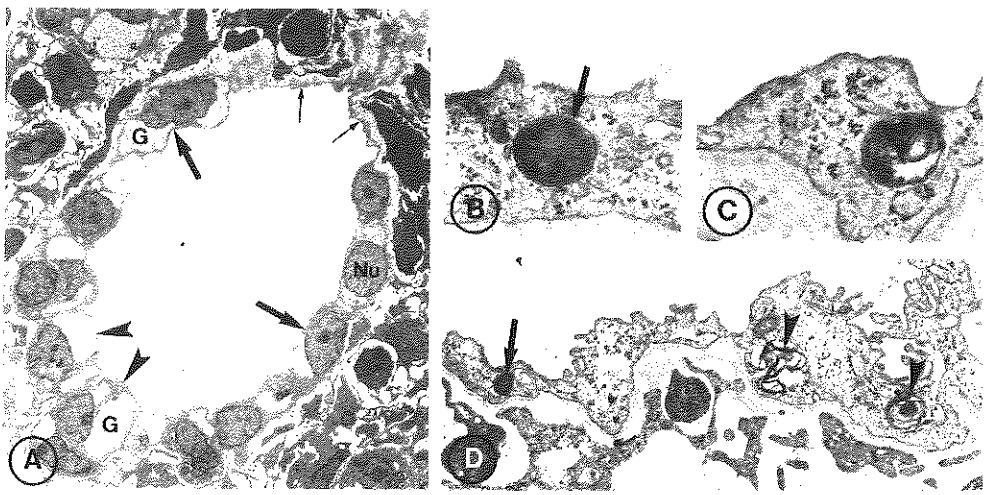
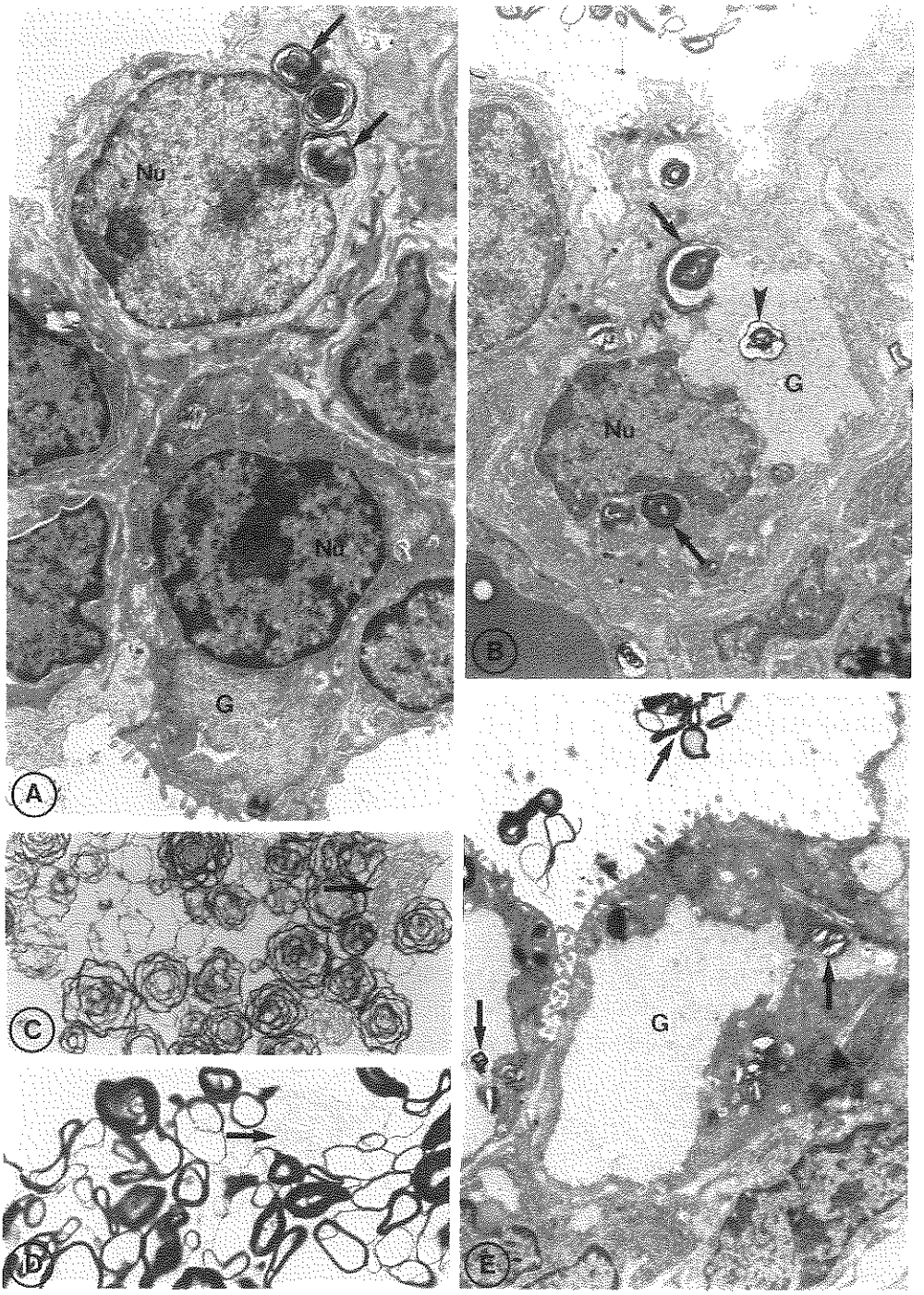


Figure 4 TEM pictures of (from type II cells) developing type I cells in control fetal rat lungs (Nitrofen-exposed group not shown); days 20 and 21. (Nu, nucleus; G, glycogen). ***(A)*** *A dilated acinar tubule, lined by cuboid type II cells (arrowheads), low cuboid cells with an extended basis (arrows), and some thinner cells (thin arrows). Large glycogen fields are above or below the nucleus in cuboid cells, and beside the nucleus in low cuboid cells. 1.100x.* ***(B)*** *In cytoplasmic processes of thinner cells precursors of MLBs such as dense bodies (arrow) can be found. 26.000x.* ***(C)*** *An osmiophilic MVB present in a thinner cell. 26.000x.* ***(D)*** *A thinner cell, containing a dense body (arrow) and two lamellar bodies (arrowheads). 9.200x.*

In the Nitrofen-exposed group (Figs. 5B,D) 14 out of 15 fetuses (day 19-22) had "MLBs" with higher electron density and confluent thick lamellae, whereas in the control group (Figs. 5A,C,E) only 5 out of 13 fetuses (day 19-22) showed this picture. "MLBs" with such a rather unusual appearance were observed both intra- and extracellularly. However, extracellular "MLBs" were more obviously different (Figs. 5C,D). Within this abnormal-looking extracellular material more or less normal tubular myelin was present.

Figure 5 TEM pictures of more or less mature type II cells and extracellular MLBs in control (a,c,e) and Nitrofen-exposed (b,d) fetal rat lungs; day 22. (Nu, nucleus; G, glycogen). ***(A)*** *Mature type II cells with some MLBs (arrows); glycogen fields are frequently absent. 6.000x.* ***(B)*** *Nitrofen-exposed fetal rat lung. Note the LB within the glycogen field (arrowhead) and the confluent thick lamellae of the other MLBs (arrows). 6.000x.* ***(C)*** *Extracellular MLBs and tubular myelin (arrow). 9.000x.* ***(D)*** *Nitrofen-exposed group; extracellular MLBs with a rather unusual appearance, i.e. dense and confluent (Cf. c). Some tubular myelin is present (arrow). 9.000x.* ***(E)*** *Control group; a large glycogen field and electron dense MLBs; in some control lungs MLBs show confluent thick lamellae as well (arrows). 6.000x.*


Nu
Nu
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Phospholipids in bronchoalveolar lavage fluid

In Nitrofen-exposed fetuses leakage of fluid from the lung during bronchoalveolar lavage occurred sooner than in control fetuses, and the infused volume was not easily recovered again. By this, broncholalveolar lavage fluid from Nitrofen-exposed lungs was difficult to compare quantitatively to that from control lungs. Anyhow, it yielded significantly lower amounts of phospholipids than lavage fluid from control lungs. However, the quantity of individual phospholipids expressed as percentages of the total amount (Table 1) did not show significant differences between Nitrofen-exposed and control lungs.

Table 1 *Phospholipid fractions in bronchoalveolar lavage fluid from Nitrofen-exposed and control fetal (day 22) rats.*

Phospholipids	Control (n=10; 5 pools)	Nitrofen (n=13; 4 pools)
Total (nmol/fetus)	129.1 ± 81.9	39.3 ± 60.4
Lysophosphatidylcholine (%)	0.0 ± 0.0	0.1 ± 0.2
Sphingomyelin (%)	2.1 ± 0.4	2.0 ± 0.9
Phosphatidylcholine (%)	81.0 ± 3.3	82.5 ± 5.1
Phosphatidylinositol (%)	2.7 ± 0.7	2.1 ± 0.8
Phosphatidylethanolamine (%)	1.5 ± 0.3	1.1 ± 0.2
Phosphatidylglycerol (%)	7.7 ± 1.9	7.8 ± 2.3
Other minor phospholipids (%)	1.5 ± 0.9	1.9 ± 0.9

SP-A in bronchoalveolar lavage fluid

Dot blot immunoassays with serial dilutions of bronchoalveolar lavage fluid from Nitrofen-exposed and control fetal rats (Fig. 6) showed no difference in SP-A-reactivity per nmol phospholipid. Thus, the relative amount of SP-A in the lavaged surfactant was the same in both groups. It showed a similar positive reactivity pattern as SP-A in bronchoalveolar lavage fluid from patients with alveolar proteinosis (Fig. 6). The controls (normal mouse, human, and rat serum) were negative (Fig. 6).

3.5 Discussion

Ultrastructure of alveolar epithelial cells in hypoplastic lungs

The epithelial cells that line the prospective pulmonary acinus in hypoplastic and control fetal rat lungs, have all the characteristics of (im)mature type II alveolar epithelial cells. These cells are low columnar to approximately cuboid in shape, and display a large and

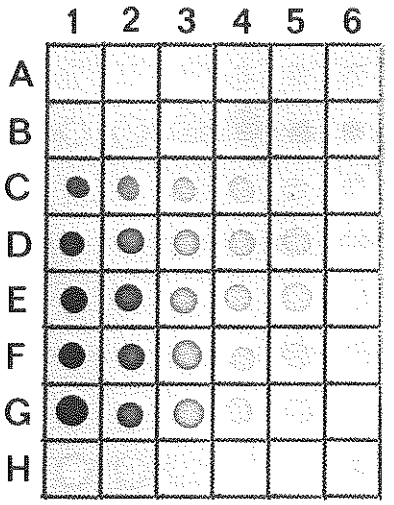

Figure 6 Dot blot assay, incubated with anti-SP-A antiserum (1:400). Lanes 1-6: serial dilutions 1/10-1/320. Lane A: normal mouse serum. B: normal human serum. C: broncho-alveolar lavage fluid (BAL; 1:5) from patients with alveolar proteinosis. D and E: BAL (pellet) from control rats. F and G: BAL (pellet) from Nitrofen-exposed rats. H: normal rat serum. Note similar staining patterns for BAL from control and Nitrofen-exposed rat fetuses.

roundish nucleus, and frequently large glycogen fields in the apical or basal cell parts. As shown previously for the rat (Brandsma et al. 1992; Otto-Verberne and Ten Have-Opbroek 1987), such cells also exhibit a cytoplasmic staining for SP-A, another distinctive criterium for type II cell recognition (Ten Have-Opbroek 1975, 1979). The same features have been described for fetal type II cells in the mouse (Ten Have-Opbroek 1975, 1979, 1981), the human (Otto-Verberne et al. 1988), and the Rhesus monkey (Ten Have-Opbroek and Plopper 1992). Our study also demonstrates that type II cells in hypoplastic and control fetal rat lungs contain a variety of IBs, namely cytoplasmic IBs, MVBs, DBs, LBs and MLBs. The former four IBs highly probably are precursory stages of MLBs (Ten Have-Opbroek 1988; Ten Have-Opbroek et al. 1990). Furthermore, we found a decrease in glycogen content during maturation. This finding is in line with earlier reports for several species that describe that maturation of type II cells goes along with a marked decrease in glycogen content and a concomitant increase in the number of IBs, particularly MLBs (Chi 1985; Kikkawa and Spitzer 1969; Ten Have-Opbroek et al. 1990). This course of events is not surprising, because glycogen may constitute a source of energy as well as a source of substrate for lipid bilayers of the cell. Evidence that in type II cells glycogen may also specifically provide substrates for the production of surfactant phospholipid biosynthesis has been obtained in biochemical studies performed in the developing rat lung (review: Batenburg 1992).

A remarkable finding in our study is the existence of unusual looking "MLBs" characterized by a higher electron density and confluent thick lamellae. In the Nitrofen-exposed group 14 out of 15 fetuses show this picture, whereas in the control group only 5 out of 13 fetuses do so. The reason for this phenomenon, not mentioned by other authors,

is not clear but suggests a qualitative difference in surfactant composition. Williams et al. (1991) show that changes in morphological appearance of surfactant phospholipids can be produced by adding SP-A to the lipid mixture. Therefore, we have included further research concerning surfactant composition (see below).

Besides cuboid type II cells, hypoplastic and control fetal rat lungs also display lower cell types from day 20 onward. These cells must represent from type II cells developing type I cells with intermediate characteristics (Adamson and Bowden 1975; Brandsma et al. 1992b; Ten Have-Opbroek 1981, 1991). The developing type I cells occur in various forms, i.e., as low cuboid cells with an extended basis or as thinner cells; both forms contain precursory stages of MLBs and frequently large glycogen fields. Initially, these glycogen fields are predominantly located in the lateral cytoplasmic compartments. Further attenuation of these cells goes along with a further decrease in IB and glycogen content. Very thin cells, i.e. mature type I cells seen on day 22, lack IBs and glycogen. There are no ultrastructural differences between developing or mature type I cells in hypoplastic and control lungs.

In summary, the major differences between hypoplastic and control fetal rat lungs are that there are less mature MLBs on day 20 and more glycogen on day 22 in type II cells of hypoplastic lungs, suggesting that these cells in hypoplastic lungs are retarded in maturation compared to those in control lungs. In a former study (Brandsma et al. 1992a) we showed that Nitrofen-exposed hypoplastic fetal rat lungs are retarded with respect to the differentiation of type II into type I cells, based on immunofluorescence reactivity for SP-A. Apparently, the maturation of immature type II cells to mature type II cells is retarded as well. This finding suggests that the degree of maturity of type II cells may play a role in type I cell differentiation.

Only a few other electron microscopical studies have been performed, using the rat model for CDH and pulmonary hypoplasia. Kimbrough et al. (1974) report no ultrastructural abnormalities of epithelial cells in hypoplastic lungs of newborn rats. However, in an abstract of the same group (Kimbrough et al. 1973) poor expansion of the lungs in combination with less well developed LBs is mentioned. We have also found retardation of development of the future air spaces in lungs from Nitrofen-exposed fetuses (Brandsma et al. 1992a). From their (Kimbrough et al. 1973) results it is not clear whether the less well developed LBs are immature or abnormally formed LBs, as we have found in the present study. Studies in the fetal lamb model for CDH and pulmonary hypoplasia, in which CDH is created surgically during the pseudoglandular phase of lung development (Hashimoto et al. 1985; Pringle 1989), mention an abundance of mature type II cells, based on the presence of mature MLBs. On the other hand, in the fetal lamb model in which pulmonary hypoplasia is achieved by phrenic nerve section (Nagai et al. 1988) much more glycogen and a significantly smaller number of LBs have been found in type II cells. Thus, according to this study, there is an abundance of immature type II cells. In human lungs from children with CDH one study (Nakamura et al. 1991) also describes a

reduced number of LBs in type II cells in the affected lungs in four out of five cases examined.

In short, data in the literature on ultrastructural differences between alveolar epithelial cells in hypoplastic and control lungs are scarce and contradictory. We conclude from the studies in our fetal rat model of pulmonary hypoplasia and CDH that both categories of lungs display similar ultrastructural features, although with an apparently different time scale and abundance, as a result of which hypoplastic lungs look less mature near term than control lungs.

Surfactant - phospholipids and SP-A - in hypoplastic lungs

In view of our finding of abnormal looking "MLBs" we have measured phospholipid fractions and SP-A-content in bronchoalveolar lavage fluid from Nitrofen-exposed and control animals. The same amount of SP-A per mol phospholipid appears to be present in both groups. The same holds for the relative amounts of phospholipid components: there is no difference between the Nitrofen-exposed group and the control group. The phospholipid composition in bronchoalveolar lavage fluid from these groups is roughly the same as described for normal newborn, young, and adult rats (Egberts et al. 1992). However, the total amount of phospholipid (nmol) recovered from Nitrofen-exposed rat fetuses by bronchoalveolar lavage is significantly less than that recovered from control rats. In our opinion, this does not prove that these Nitrofen-exposed hypoplastic fetal rat lungs are surfactant deficient. Electron microscopically, no obvious difference is observable in the abundance of intra- and extracellular MLBs between Nitrofen-exposed and control lungs on day 22. Very likely, the difference found in total amounts of phospholipid is largely due to a difference in bronchoalveolar lavage efficiency, because hypoplastic lungs could not be washed as efficiently as normal lungs. Another factor could be the difference in weight between hypoplastic and control lungs. Therefore, correction of the total amount of phospholipids for lung weight may reduce the diverging results. Bronchoalveolar lavage is a good method to obtain material from normal and hypoplastic lungs for *qualitative* analysis of phospholipids and proteins. For quantitative measurements, whole lung homogenates or preferably, MLB-content may be more suitable materials.

Findings on surfactant synthesis from former studies using the rat model (Kavlock et al. 1982; Kimbrough et al. 1973; Stone and Manson 1981; Suen et al. 1992; Tovar et al. 1992) are divergent. Kavlock et al. (1982), Kimbrough et al. (1973) and Suen et al. (1992) suggest a decrease in surfactant synthesis, Stone and Manson (1981) find no effect of Nitrofen, while Tovar et al. (1992) describe an increase in surfactant synthesis. Kavlock et al. (1982) mention decreased total dipalmitoyl phosphatidylcholine (DPC) per lung and decreased total sphingomyelin (SPH) per lung on day 21 after Nitrofen exposure. From these results it is not clear whether DPC and SPH per mg lung tissue are decreased as well. Suen et al. (1992) has studied two Nitrofen-exposed groups on day 21: one with

and one without a defect in the diaphragm. They have found less disaturated phosphatidylcholine per lung and per μg DNA in the CDH group than in the group without CDH. On the other hand, Stone and Manson (1981) have found no difference in 14C-choline uptake, cholinephosphotransferase activity and phospholipids per mg lung tissue between Nitrofen-exposed and control fetuses on days 20 and 21. Tovar et al. (1992) have even found increased levels of PC, PG and PI on day 21 in Nitrofen-exposed fetuses, compared to normal controls. In summary, the findings on surfactant-synthesis in hypoplastic rat lungs are not uniform at all. In general, in most studies surfactant synthesis is found to be either not affected or decreased after Nitrofen exposure. Only one study (Tovar et al. 1992) describes an increase in surfactant synthesis.

A similar controversy can be observed in other animal models for pulmonary hypoplasia, and in humans. Using the fetal lamb model for CDH and pulmonary hypoplasia, only one study documents the status of the surfactant system (Glick et al. 1992) and mentions decreased total phospholipids and increased proteins per g lung tissue. In addition, the relative amounts of different phospholipid components is said to be altered; the proportion of PC is significantly reduced, whereas that of LPC and PI are significantly increased. These findings are not confirmed by the present study. In hypoplastic rat lungs associated with oligohydramnion (Blachford and Thurlbeck 1987) reduced amounts of phospholipid per lung have been found on day 21, whereas the amount per mg dry lung weight is the same as in normal control lungs. In experiments with lungs made hypoplastic by cervical spinal cordotomy or phrenic nerve section (Kitterman 1984) no difference has been found in DSPC concentration in lung tissue or lung lavage fluid. Human case studies report decreased L/S ratios in amniotic fluid (Berk and Grundy 1982; Hisanaga et al. 1984) as well as decreased phospholipids per μg DNA (Blackburn et al. 1977; Nakamura et al. 1991; Wigglesworth et al. 1981). Small clinical trials of surfactant instillation (either prophylactic, or therapeutic; Bos et al. 1991; Glick et al. 1991; Lotze et al. 1993) show varying results.

From the results in our model for pulmonary hypoplasia and CDH, we conclude that in hypoplastic fetal rat lungs type II cells are less mature than in control lungs; nevertheless, the composition of extracellular surfactant is likely the same.

3.6 **Acknowledgments**

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3.7 References

Adamson IYR, Bowden DH: Derivation of type 1 epithelium from type 2 cells in the developing rat lung. Lab Invest 32:736-745, 1975.

Askenazi SS, Perlman M: Pulmonary hypoplasia: lung weight and radial alveolar count as criteria of diagnosis. Arch Dis Child 54:614-618, 1979.

Batenburg JJ: Surfactant phospholipids: synthesis and storage. Am J Physiol 262:L367-L385, 1992.

Berk C, Grundy M: 'High risk' lecithin/sphingomyelin ratios associated with neonatal diaphragmatic hernia. Case reports. Br J Obstet Gynaecol 89:250-251, 1982.

Blachford KG, Thurlbeck WM: Lung growth and maturation in experimental oligohydramnios in the rat. Pediatr Pulmonol 3:328-333, 1987.

Blackburn WR, Logsdon P, Alexander JA: Congenital diaphragmatic hernia: studies of lung composition and structure. Am Rev Resp Dis 115(Suppl):275, 1977.

Bos AP, Tibboel D, Hazebroek FWJ, Molenaar JC, Lachmann B, Gommers D: Surfactant Replacement Therapy in High-Risk Congenital Diaphragmatic Hernia. Lancet 338:1279, 1991.

Brandsma AE, Ten Have-Opbroek AAW, Vulto IM, Molenaar JC, Tibboel D: Alveolar epithelial composition and architecture in a rat model of lung hypoplasia. Am Rev Resp Dis 145(Suppl):A126, 1992a.

Brandsma AE, Ten Have-Opbroek AAW, Vulto IM, Molenaar JC, Tibboel D: Type I cell differentiation and surfactant protein SP-A reactivity in fetal rat lung. Am Rev Resp Dis 145(Suppl):A131, 1992b.

Chi EY: The ultrastructural study of glycogen and lamellar bodies in the development of fetal monkey lung. Exp Lung Res 8:275-289, 1985.

Egberts J, Sprengers BM, Sietaram MA: Comparison of the pulmonary surfactant content in alveolar macrophages of newborn, young, and adult rats. Exp Lung Res 18:275-285, 1992.

Fiske CH, Subbarow Y: The colorimetric determination of phosphorus. J Biol Chem 64:375-400, 1925.

Glick PL, Leach CL, Besner GE, Morin FC, Malanowska-Kantoch A, Robinson LK, Brody A, Lele AS, Holm B, Rodgers BT, Msall ME, Allen JE, Jewett TC, Cooney DR: Pathophysiology of congenital diaphragmatic hernia. III: Exogenous Surfactant Therapy (EST) for the high risk neonate with CDH. Am Rev Resp Dis 143(Suppl):A308, 1991.

Glick PL, Stannard VA, Leach CL, Rossman J, Hosada Y, Morin FC, Cooney DR, Allen JE, Holm B: Pathophysiology of congenital diaphragmatic hernia II: the fetal lamb model is surfactant deficient. J Pediatr Surg 27:382-388, 1992.

Hashimoto EG, Pringle KC, Soper RT, Brown CK: The creation and repair of diaphragmatic hernia in fetal lambs: morphology of the type II alveolar cell. J Pediatr Surg 20:354-356, 1985.

Hisanaga S, Shimokawa H, Kashiwabara Y, Maesato S, Nakano H: Unexpectedly low lecithin/sphingomyelin ratio associated with fetal diaphragmatic hernia. Am J Obstet Gynecol 149:905-906, 1984.

Kavlock RJ, Chernoff N, Rogers E, Whitehouse D, Carver B, Gray J, Robinson K: An analysis of fetotoxicity using biochemical endpoints of organ differentiation. Teratology 26:183-194, 1982.

Kikkawa Y, Spitzer R: Inclusion bodies of type II alveolar cells: species differences and morphogenesis. Anat Rec 163:525-541, 1969.

Kimbrough RD, Gaines TB, Linder RE: The effect of 2,4-Dichlorophenyl-p-nitrophenyl ether on the rat fetus. Toxicol Appl Pharmacol 25:456, 1973.

Kimbrough RD, Gaines TB, Linder RE: 2,4-Dichlorophenyl-p-nitrophenyl ether (TOK): effects on the lung maturation of rat fetus. Arch Environ Health 28:316-320, 1974.

Kitterman JA: Fetal lung development. J Dev Physiol 6:67-82, 1984.

Kluth D, Kangah R, Reich P, Tenbrinck R, Tibboel D, Lambrecht W: Nitrofen-induced diaphragmatic hernias in rats: an animal model. J Pediatr Surg 25:850-854, 1990.

Lau C, Cameron AM, Irsula O, Antolick LL, Langston C, Kavlock RJ: Teratogenic effects of nitrofen on cellular and functional maturation of the rat lung. Toxicol Appl Pharmacol 95:412-422, 1988.

Lotze A, Knight GR, Martin GR, Bulas DI, Hull WM, O'Donnell R, Whitsett JA, Short BL: Improved pulmonary outcome after exogenous surfactant therapy for respiratory failure in term infants requiring ECMO. J Pediatr 122:261-268, 1993.

Manson JM: Mechanism of Nitrofen teratogenesis. Environ Health Persp 70:137-147, 1986.

Molenaar JC, Bos AP, Hazebroek FWJ, Tibboel D: Congenital diaphragmatic hernia, what defect? J Pediatr Surg 26:248-254, 1991.

Nagai A, Thurlbeck WM, Jansen AH, Ioffe S, Chernick V: The effect of chronic biphrenectomy on lung growth and maturation in fetal lambs. Morphologic and morphometric studies. Am Rev Respir Dis 137:167-172, 1988.

Nakamura Y, Yamamoto I, Fukuda S, Hashimoto T: Pulmonary acinar development in diaphragmatic hernia. Arch Pathol Lab Med 115:372-376, 1991.

Oomen LCJM, Ten Have-Opbroek AAW, Hageman PC, Oudshoorn-Snoek M, Egberts J, van der Valk MA, Calafat J, Demant P: Fetal mouse alveolar type II cells in culture express several type II cell characteristics found in vivo, together with major histocompatibility antigens. Am J Respir Cell Mol Biol 3:325-339, 1990.

Otto-Verberne CJM, Ten Have-Opbroek AAW: Development of the pulmonary acinus in fetal rat lung: a study based on an antiserum recognizing surfactant-associated proteins. Anat Embryol 175:365-373, 1987.

Otto-Verberne CJM, Ten Have-Opbroek AAW, Balkema JJ, Franken C: Detection of the type II cell or its precursor before week 20 of human gestation, using antibodies against surfactant-associated proteins. Anat Embryol 178:29-39, 1988.

Pringle KC: Lung development in congenital diaphragmatic hernia. In: Puri P (ed.) Congenital Diaphragmatic Hernia. Mod Probl Paediatr, Basel, Karger 24:28-53, 1989.

Stone LC, Manson JM: Effects of the herbicide 2,4-dichlorophenyl-p-nitrophenyl ether (nitrofen) on fetal lung development in rats. Toxicology 20:195-207, 1981.

Suen HC, Catlin EA, Ryan DP, Donahoe PK: Biochemical evidence of lung immaturity in neonatal rats with congenital diaphragmatic hernia created by maternal feeding of Nitrofen. In: Proceedings of the 23rd Annual Meeting, American Pediatric Surgical Association, Colorado Springs, Colorado, May 13-16, page 36, 1992.

Tenbrinck R, Tibboel D, Gaillard JLJ, Kluth D, Bos AP, Lachmann B, Molenaar JC: Experimentally induced congenital diaphragmatic hernia in rats. J Pediatr Surg 25:426-429, 1990.

Ten Have-Opbroek AAW: Immunological study of lung development in the mouse embryo: appearance of a lung-specific antigen, localized in the great alveolar cell. Dev Biol 46:390-403, 1975.

Ten Have-Opbroek AAW: Immunological study of lung development in the mouse embryo: II. First appearance of the great alveolar cell, as shown by immunofluorescence microscopy. Dev Biol 69:408-423, 1979.

Ten Have-Opbroek AAW: The development of the lung in mammals: an analysis of concepts and findings. Am J Anat 162:201-219, 1981.

Ten Have-Opbroek AAW: Lung development in the mouse embryo. Exp Lung Res 17:111-130, 1991.

Ten Have-Opbroek AAW, Dubbeldam JA, Otto-Verberne CJM: Ultrastructural features of type II alveolar epithelial cells in early embryonic mouse lung. Anat Rec 221:846-853, 1988.

Ten Have-Opbroek AAW, Otto-Verberne CJM, Dubbeldam JA: Ultrastructural characteristics of inclusion bodies of type II cells in late embryonic mouse lung. Anat Embryol 181:317-323, 1990.

Ten Have-Opbroek AAW, Plopper CG: Morphogenetic and functional activity of type II cells in early fetal Rhesus monkey lungs: a comparison between primates and rodents. Anat Rec 234:93-104, 1992.

Touchstone JC, Chen JC, Beaver KM: Improved separation of phospholipids in thin layer chromatography. Lipids 15:61-62, 1980.

Tovar JA, Alfonso LF, Sasieta MJ, Ingunza N, Alvarez FJ, Vallis i Soler A: Lung phospholipids in experimental congenital diaphragmatic hernia. In: Proceedings of the 7th International Workshop of Surfactant Replacement, San Sebastian, Spain, June 1-2, page 63, 1992.

Wenstrom KD, Weiner CP, Hanson JW: A Five-Year Statewide Experience with Congenital Diaphragmatic Hernia. Am J Obstet Gynecol 165:838-842, 1991.

Wigglesworth JS, Desai R, Guerrini P: Fetal lung hypoplasia: biochemical and structural variations and their possible significance. Arch Dis Child 56:606-615, 1981.

Williams MC, Hawgood S, Hamilton RL: Changes in lipid structure produced by surfactant proteins SP-A, SP-B, and SP-C. Am J Respir Cell Mol Biol 5:41-50, 1991.

Chapter 4

THE NATURAL HISTORY OF CONGENITAL DIAPHRAGMATIC HERNIA AND PULMONARY HYPOPLASIA IN THE EMBRYO

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THE NATURAL HISTORY OF CONGENITAL DIAPHRAGMATIC HERNIA AND PULMONARY HYPOPLASIA IN THE EMBRYO

4.1 Abstract

Up to now, descriptions of the natural history of congenital diaphragmatic hernia (CDH) associated with pulmonary hypoplasia are based exclusively on observations made in the fetal period. However, nothing is known about the events that take place in an embryo with CDH. Recently, an animal model of CDH and PH has been established in rat embryos to study the embryology and natural history of this lesion. We exposed 36 pregnant Sprague-Dawley rats to a single dose of 100 mg Nitrofen on day 11 of pregnancy. A total of 356 staged embryos and fetuses from day 13 to day 21 were studied by light and scanning electron microscopy. The litters of 9 untreated rats (124 normal age-matched embryos and fetuses) served as controls. The abnormal development of the diaphragmatic anlage was first seen in embryos aged 13 to 14 days. A defect appeared in the dorsal part of the diaphragm, normally on the right side. The liver grew through this defect early on. Gut was found in an intrathoracic position only in the very late stages (day 21/22) and newborns. Compared to controls, lungs of Nitrofen-exposed embryos with CDH were smaller, depending on the size of liver found in the thoracic cavity. Histologically, compression of lung was absent at these stages. Most authors speculate that CDH results because the pleuroperitoneal canals fail to close at the end of the embryonic period (i.e. week 8 to 10 in human development) leading to a defect in the dorsolateral region of the diaphragm. However, contradictory to this assumption, our findings indicate that diaphragmatic defects develop in early embryonic life. They are easy to identify even in rat embryos as early as 14 days. The early ingrowth of liver into the thoracic cavity through these defects is the crucial step in the pathogenesis of pulmonary hypoplasia in CDH, because it is the liver and not the gut that reduces the thoracic cavity in the embryo. Normal lung growth will be hampered in early embryonic stages according to the size of the liver mass inside the thoracic cavity. Recent reports suggest that the presence of liver in the fetal thoracic cavity affects the outcome of a fetus with CDH. This observation is in accordance with our findings, suggesting that the major events of pathogenesis take place in the embryo and not in the fetus, as often assumed.

4.2 Introduction

The outcome of a newborn with congenital diaphragmatic hernia (CDH) depends on the severity of the associated pulmonary hypoplasia. Most authors consider lung hypoplasia as a secondary abnormality: delayed or incomplete closure of the so-called pleuroperitoneal canals may result in the herniation of intestine into the thoracic cavity leading to abnormal compression of the developing lungs (Gray and Skandalakis 1972; Wells 1954). Studies on the natural history of CDH in humans and data derived from experimental animal models (Adzick et al. 1985; DeLorimier et al. 1976; Harrison et al. 1980a and 1980b) seem to support this assumption. These studies, however, are based exclusively on observations made in the fetal period while data that may elucidate the natural history of CDH in the embryonic period are not at hand. An animal model of CDH and pulmonary hypoplasia has been recently established in rat embryos using Nitrofen (2,4-dichlorophenyl-p-nitro phenyl ether) as teratogen (Kluth et al. 1990; Tenbrinck et al. 1990). We used this model to define the specific morphological changes of CDH and pulmonary hypoplasia during the embryogenesis of this lesion.

4.3 Materials and Methods

Adult female Sprague-Dawley rats weighing between 196 and 319 g (mean: 261 g), were bred under normal laboratory conditions. After controlled overnight mating, pregnancy was verified by means of the vaginal smear method. The day of positive smear was rated as day zero of pregnancy. The pregnant rats were exposed to a single dose (100 mg/rat) of orally administered Nitrofen (Wako Chemicals, Neuss, Germany) on day 11 of pregnancy. The substance was mixed with 20 g of moistened commercial rat chow (Altromin, Lage, Germany) and offered to the rats after a 24-h period of fasting. Rats not consuming the mixture within 12 hours were excluded. Using this method, 36 pregnant rats could be exposed successfully to Nitrofen. Thereafter the rats were again supplied with food and water ad libitum. Nine other rats, also undergoing a 24-hour fast, served as controls.

Four rats from the Nitrofen group plus one control rat were killed on each day between day 13 and 21 of pregnancy. A total of 480 embryos and fetuses (356 from the Nitrofen group, 124 controls) were removed by laparotomy and were then staged after Witschi (1962). One litter from each treated group and three embryos from each control rat (92 Nitrofen embryos plus 27 control embryos) were fixed in Bouin, embedded in paraffin, cut in serial sections (7 μm) and stained with hematoxilin-eosin for light microscopy (LM).

The remaining embryos and fetuses (264 Nitrofen embryos and 97 control embryos) were fixed in glutaraldehyde for 24 hours and then microdissected in 80% ethanol. After

dehydratation, the embryos were dried by critical point method, gold-sputtered, and then examined and photographed with a DMS 940 scanning electron microscope (SEM) (C.Zeiss, Oberkochen, Germany).

4.4 Results

SEM findings

In the first group (13 days) a total of 30 Nitrofen embryos and 5 embryos of the control group were studied. No deviation in the morphology of the diaphragm was found, when we compared the Nitrofen group with the control group. Furthermore, we found no differences between the Nitrofen group and the control animals when we studied the development of the lungs at this stage (Fig. 1).

In the second group (14 days) a total of 41 Nitrofen embryos and 4 controls were analyzed. In this age group there was clear evidence of disturbed diaphragmatic development in the embryos of the Nitrofen group while evidence of disturbed lung development was absent. In the majority of these embryos, a defect was found in the dorsal part of the diaphragm (Fig. 2A). All defects appeared exclusively on the right side. They resulted in an abnormal diaphragm, leaving parts of the liver uncovered. Compared to controls, the left dorsal diaphragm was found to be normal in all studied embryos.In the next two age groups (15 days and 16 days) 34 and 32 Nitrofen embryos and 7 and 6

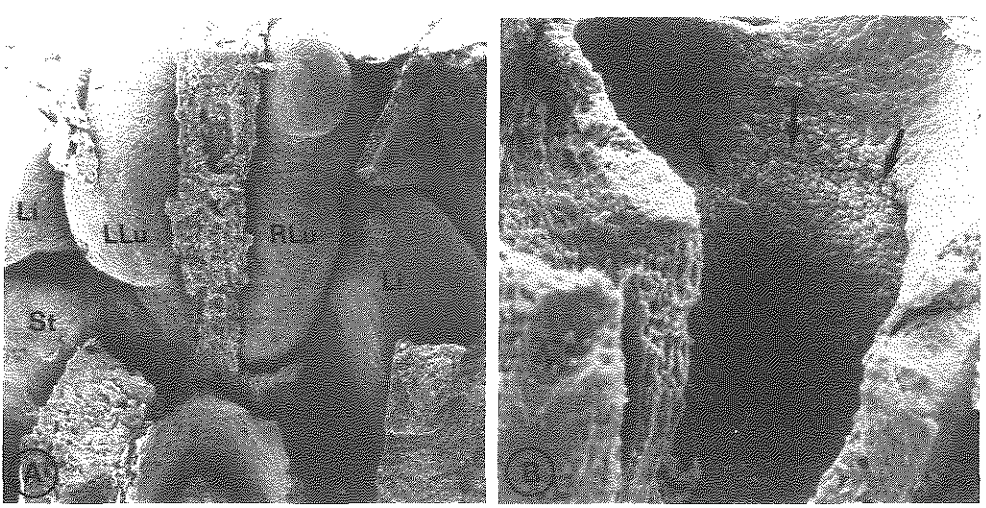

Figure 1 *SEM pictures of a 13-day-old rat embryo. This stage corresponds to a human embryo 5 weeks of age. (Li, liver; St, stomach; LLu, left lung; RLu, right lung).* ***(A)*** *Dorsal view. The lungs are still in situ.* ***(B)*** *Dorsal view into the right thoracic cavity. The right lung is removed. Arrows indicate the lower border of the diaphragm.*

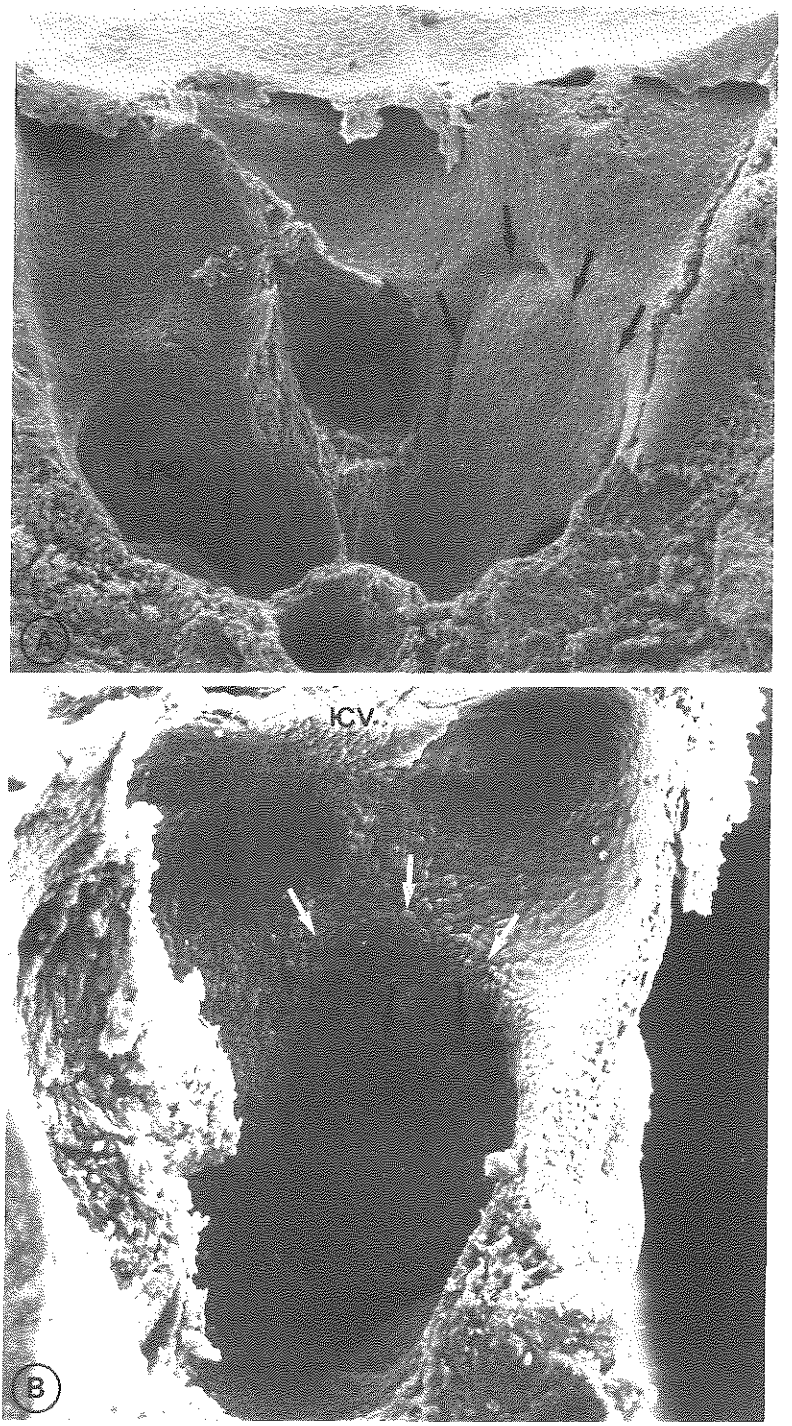

Figure 2 *SEM pictures of rat embryos. These stages correspond to a human embryo 5 to 6 weeks of age.* ***(A)*** *14-Day-old Nitrofen-exposed rat embryo. Dorsal view. The lungs are removed. The left pleuroperitoneal opening (LPO) is normal. A large defect is seen in the right diaphragm (black arrows). The diaphragm ends abnormally high, leaving parts of the liver oncovered.* ***(B)*** *13.5-Day-old rat embryo (control group). Black arrows indicate the border of the normally developed diaphragm. White arrows indicate the site of this border in a 13-day-old embryo. The downgrowth of the diaphragm is notable. (ICV, inferior vena cava).*

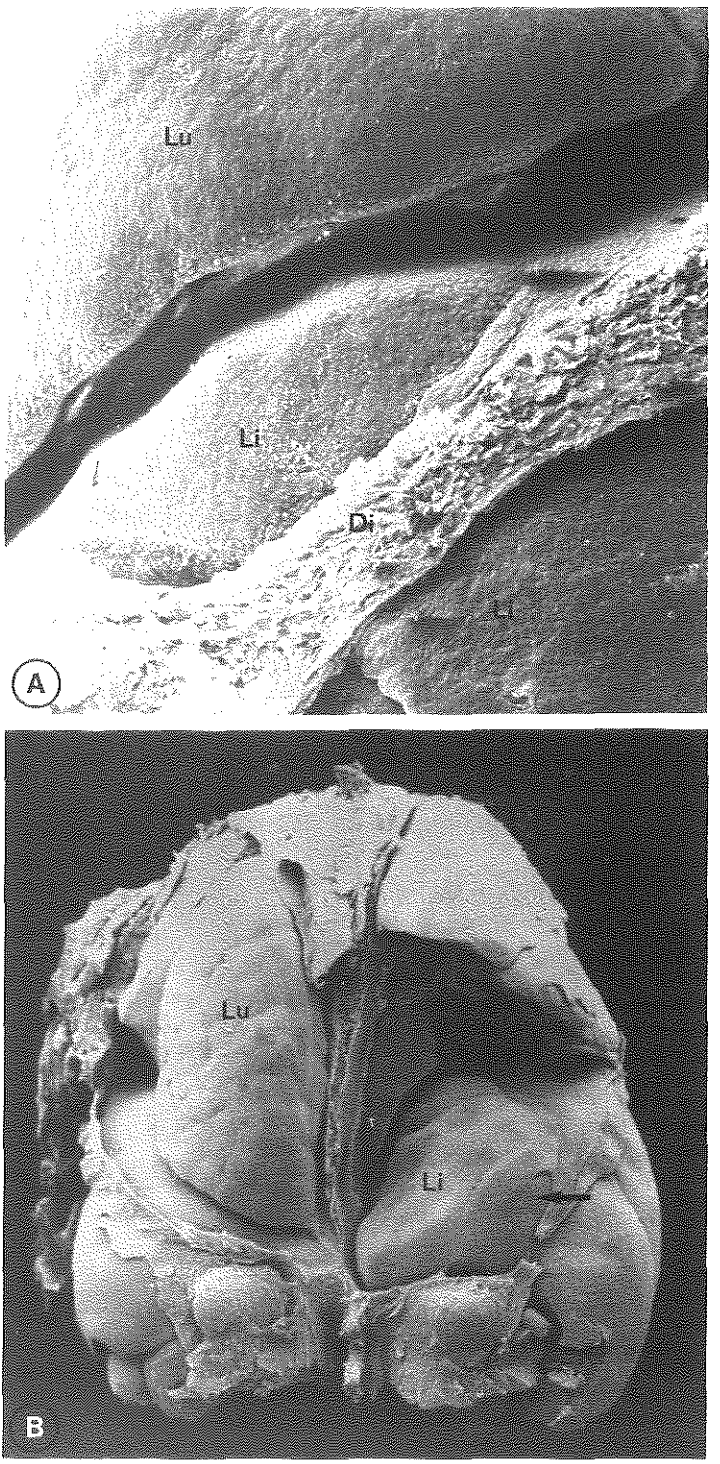

Figure 3 *SEM pictures of 16-day-old Nitrofen-exposed rat embryos. This stage corresponds to a human embryo 7 weeks of age. (Li, liver; Lu, lung; Di, diaphragm).*
(A) *Lateral view. A small part of the liver protrudes through the diaphragmatic defect and is now inside the thoracic cavity. Note the close contact between liver and lung.*
(B) *Dorsal view. The right lung is partially removed. The large defect with the intrathoracally displaced portion of the liver is seen. Due to the ingrowth of liver, the opening of the right pleuroperitoneal "canal" fails to close (arrow). Note that this opening represents only a small part of the whole defect.*

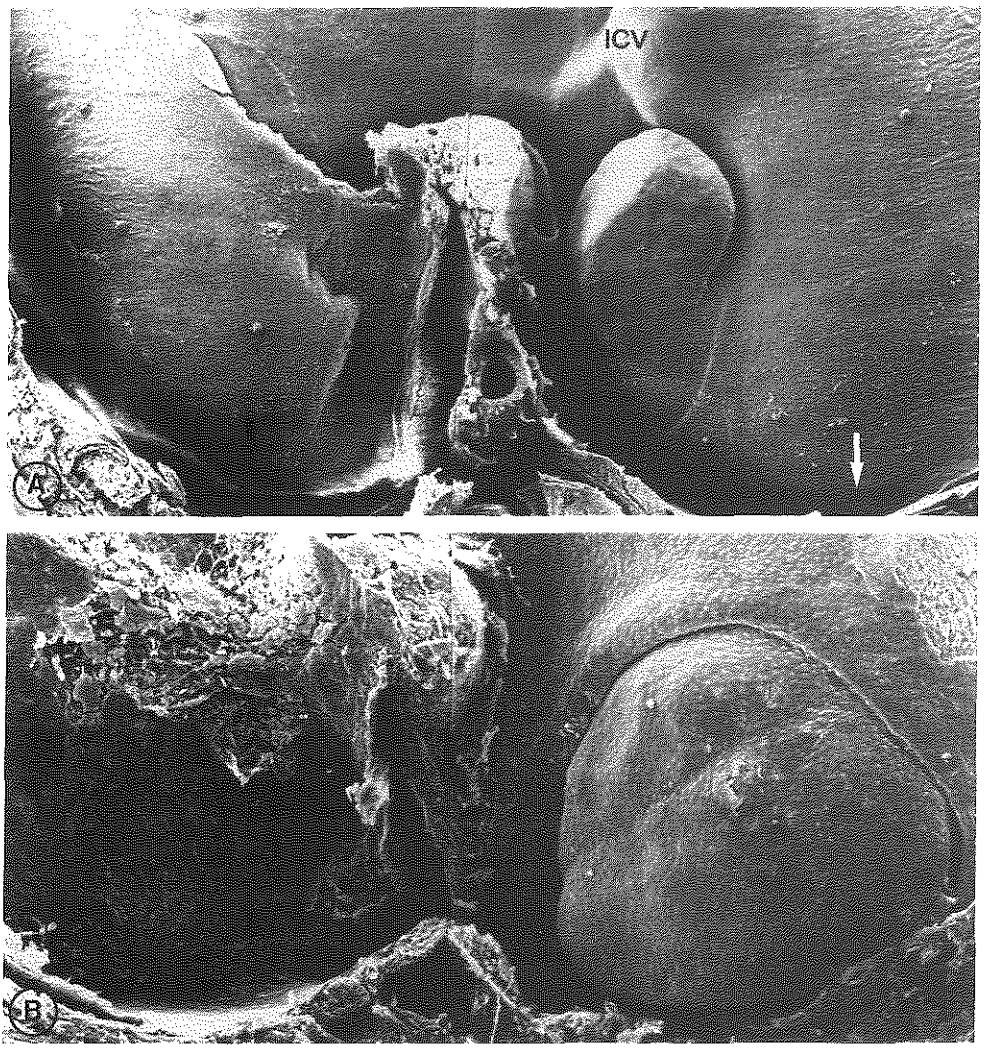

Figure 4 *SEM pictures of diaphragmatic defects. This stage corresponds to a human embryo 8 weeks of age. (ICV, inferior vena cava).* ***(A)*** *A small defect. Typically, this is in close contact with the inferior vena cava and is thus more centrally located. The white arrow indicates the region where the opening of the right pleuroperitoneal "canal" has closed.* ***(B)*** *A large defect. This defect incorporates the dorsolateral region (Bochdalek type).*

age-matched controls were studied. In most litters, the majority of embryos had a right diaphragmatic defect. In all these embryos, the liver was clearly seen inside the thoracic cavity (Fig. 3A). Two distinct forms of the malformation could be discerned. Typically, a minor form of the defect was found centrally, close to the inferior vena cava (Fig. 4A). In these cases the so-called pleuroperitoneal canals closed properly. In the major form (Fig.

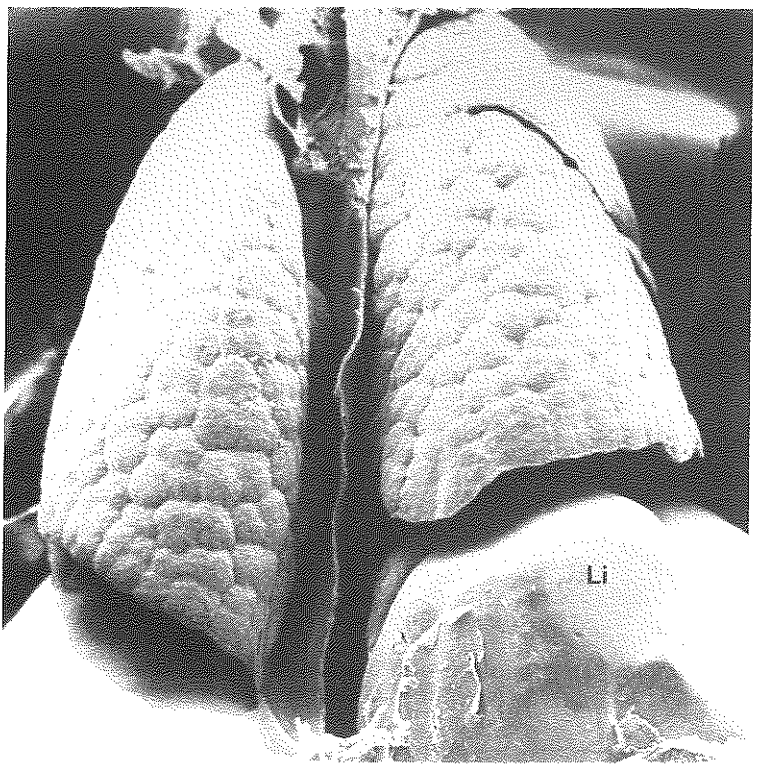

Figure 5 SEM picture of a 18-day-old Nitrofen-exposed rat embryo. This stage corresponds to a human embryo 10 weeks of age. View from dorsal. The liver (Li) occupies nearly one third of the thoracic cavity. The right lung is reduced in size. Bowel loops are absent.

4B), the defect extended from the inferior vena cava to the dorsolateral region of the diaphragm, thus incorporating the openings of the pleuroperitoneal "canals". In these age groups, the gross aspect of the lungs appeared to be normal. The lungs often covered the diaphragmatic defect, making it difficult to detect. However, after removing the lungs, the total extend of the defect became apparent (Fig. 3B). In all normal 16-day-old embryos, the openings of the pleuroperitoneal "canals" were found to be closed on the right and left side of the diaphragm. At this stage, bowel loops were still found exclusively inside the extraembryonic celom of the umbilicus.

In the following two age groups (17 days and 18 days) 28 and 25 Nitrofen embryos were compared with 7 and 8 controls. There was a remarkable difference in the growth pattern of the more "central" defects (minor forms) and the "dorsolateral" defects (major forms). In some of the embryos with a major defect, the liver occupied one third of the thoracic cavity (Fig. 5). In these cases, right lungs were markedly reduced in size compared to normal controls. In the minor defects, only a small portion of the liver reached the thorax. In these cases, the lungs were nearly normal except for a small indentation in the area of the protruding liver. In all 18-day embryos, the intestine was found inside the abdominal cavity while in the embryos of the 17-day age group, intestinal loops remained still in the extraembryonic celomic cavity of the umbilicus.

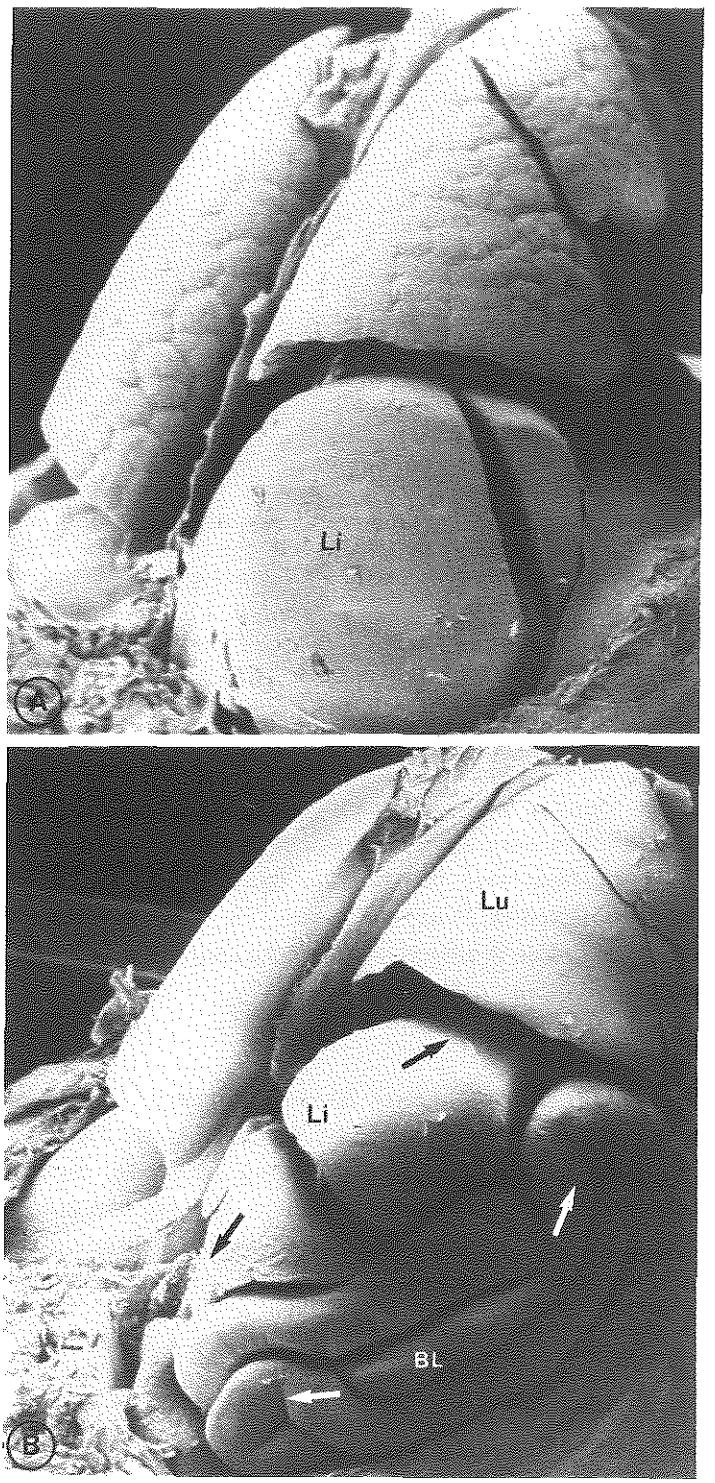

Figure 6 *SEM pictures of Nitrofen-exposed rat fetuses. These stages correspond to human fetuses older than 18 weeks. (Li, liver; BL, bowel loops; Lu, lung).* ***(A)*** *20-Day-old fetus. Half of the thoracic cavity is occupied by liver. The lung is markedly reduced. Bowel loops are still absent.* ***(B)*** *21-Day-old fetus. Bowel loops finally have entered the thoracic cavity. The liver occupies two thirds of the thoracic cavity. The lung is small.*

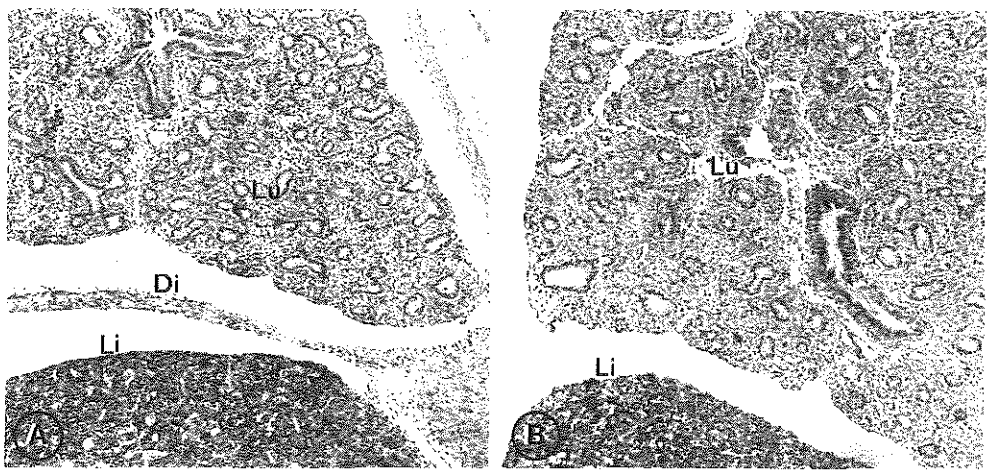

Figure 7 LM pictures of serial sections of 18-day-old rat embryos. This stage corresponds to a human embryo 10 weeks of age. (Li, liver; Lu, lung; Di, diaphragm). ***(A)*** *Control embryo.* ***(B)*** *Nitrofen-exposed embryo. Clear signs of lung compression by the ingrowing liver can not be discerned.*

The next age groups (19 days, 20 days, and 21days) represent the fetal stage of development. Here, the whole spectrum of the malformation could be observed. In some of the 19- and 20-day-old rat fetuses, over half of the chest was occupied by liver masses (Fig. 6A), while in others the ingrowth of the liver was less obvious. In those with massive liver protrusion, the lungs were small and hypoplastic while they appeared nearly normal when only small parts of liver were found inside the chest. Intrathoracally displaced bowel loops were first observed in 21-day-old fetuses (Fig. 6B). In these, the lungs were found to be reduced in size due to amount of liver occupying up to two thirds of the available thoracic space.

LM findings

Our anatomical SEM findings were confirmed by serial histological sections. The defect was first identified in 15-day-old embryos. It was always situated in the dorsal diaphragm and was found in close contact with the inferior vena cava. Starting on day 16, the liver gradually expanded into the thoracic cavity. However, clear signs of lung compression were not found in our embryos (Fig. 7). Comparison of the lungs and the thoracic cavities in age groups 16 and 18 demonstrated that in the embryo, these structures were expanding despite the ingrowth of liver (Fig. 8).

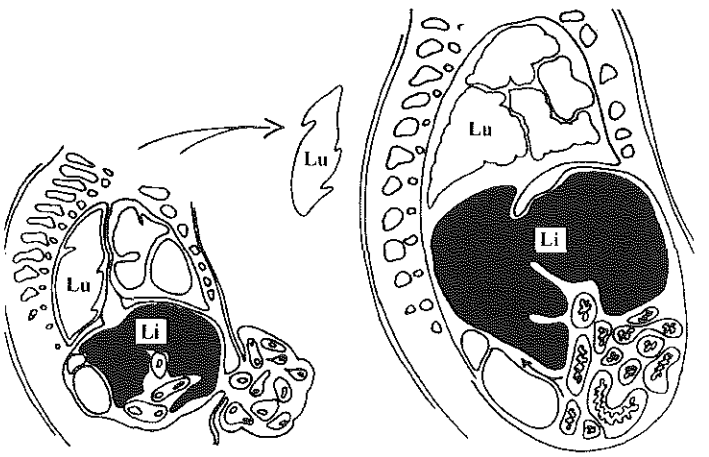

Figure 8 When two embryos of different age groups are compared (16 days versus 18 days), the expansion of the thoracic cavity becomes obvious. Thus, the lung still continues to grow, while part of the chest is occupied by liver. In a growing embryonic body, compression is unlikely. (Sketch after serial sections in same magnification).

4.5 Discussion

Since Broman's investigations (1902) on the diaphragmatic development, most authors have speculated that CDH results because the pleuroperitoneal canals fail to close at the end of the embryonic period (i.e. week 8 to 10 in human development) leading to a defect in the dorsolateral region of the diaphragm (Gray and Skandalakis 1972; Harrison 1990; Wells 1954). Bowel loops may then herniate through this defect into the chest with subsequent hypoplasia of the developing fetal lungs (Gray and Skandalakis 1972; Harrison 1990; Wells 1954). However, the natural history of this sequence of embryological and fetal events has not been previously studied. Thus, the pathogenesis of the CDH and the associated pulmonary hypoplasia is still elusive and the answers to the following questions remain unknown: (1) When does the diaphragmatic defect appear? (2) Are the pleuroperitoneal canals the precursors of the diaphragmatic defect? (3) Why is the lung hypoplastic in cases of CDH?

Since 1971, the embryotoxicity of the herbicide Nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) is well known (Ambrose et al. 1971). In 1981, Nakao et al. noticed that this teratogen can induce CDH in rats and mice. Since then, this substance has been widely used to study its effect on the lung and diaphragm (Iritani 1984; Nakao and Ueki 1987; Ueki et al. 1990). However, only the work of Iritani (1984) adresses the natural history of CDH in the embryo. In our study, we used the pulse feeding technique. Pregnant rats were exposed to a single 100-mg dose of Nitrofen on day 11 of pregnancy. This day has been previously identified by us to be highly sensitive to the teratogenetic effect of Nitrofen (Kluth et al. 1990; Tenbrinck et al. 1990).

The onset of CDH

Most authors speculate that in the human fetus with CDH, the pleuroperitoneal canals fail to close late in the embryonic period between the 8th and 10th week of gestation (Gray and Skandalakis 1972; Harrison 1990; Wells 1954). At the same time, the intestine returns into the abdominal cavity (Gray and Skandalakis 1972; Harrison 1990; Starck 1975; Wells 1954). In rats, this developmental stage is equivalent to that of 17 or 18 days of gestation. At this time, the openings of the so-called pleuroperitoneal canals are normally closed (Kluth et al. 1989).

In contrast to this assumption, it is evident from our study that the diaphragm is already malformed in the 14-day-old embryo (Fig. 2A). This age group is equivalent to that of a 5- to 6-week-old human embryo. In a 13-day-old rat embryo, signs of a disturbed diaphragmatic development are still missing (Fig. 1B). However, the onset of the malformation takes place at this stage. The diaphragmatic anlage of a 13.5-day-old embryo (Fig. 2B) is too advanced to serve as the starting point for the maldevelopment. These findings are in accordance with observations made by Iritani (1984). He found signs of disturbed diaphragmatic development in 11-day-old mouse embryos exposed to Nitrofen continuously from day 5 to 11 after conception. Thus, we conclude that in rat embryos the onset of the diaphragmatic defect clearly lies in the early embryonic period. This phase is comparable to that of a human embryo in the 5th gestational week.

The site of the defect

Iritani (1984) was the first to notice that Nitrofen-induced diaphragmatic hernias are not caused by an improper closure of the pleuroperitoneal openings but rather the result of a defective development of the so-called posthepatic mesenchymal plate (PHMP).

The observed defects were also localized in the PHMP in our SEM study (Fig. 2A). We could identify two distinct types of defects: (1) large "dorsolateral" defects and (2) small "central" defects. Large defects extended into the region of the pleuroperitoneal openings. In these cases, the closure of the pleuroperitoneal openings was usually impaired by the massive ingrowth of liver (Fig. 3B). If the defects were small, they were consistantly isolated from the pleuroperitoneal openings closing normally at the 16th or 17th day of gestation. Thus, in our embryos with CDH, the region of the diaphragmatic defect was a distinct entity and was seperated from that part of the diaphragm where the pleuroperitoneal "canals" are localized. We conclude therefore that the pleuroperitoneal openings are not the precursors of the diaphragmatic defect.

The lung in CDH:

Primary pulmonary hypoplasia

Nakao et al (1987), Ueki et al (1990) and Iritani (1984) postulated that the lung is primarily hypoplastic. In his study, Iritani even drew the conclusion that the hypoplastic lung may induce a defective PHMP which may again result in CDH (Iritani 1984).

However, in our young embryos (14 days gestational age) with a diaphragmatic defect, disorders of the early lung anlage could not be observed. This finding is in sharp contrast to previously published results by Ueki et al. (1990). They observed reduced lung growth in young mouse embryos continuously exposed to Nitrofen from postconceptual days 5 to 11. However, this reduced lung growth may be the result of Nitrofen interference with the very early lung anlage. It is well known that developing embryonic organs have specific periods of greatest sensitivity to teratogens. Continuous administration of Nitrofen for a longer period must thus result in a combination of malformations. In our study, we avoided this combination by pulse feeding of Nitrofen on day 11. The diaphragm has proved to be highly sensitive to the teratogenetic action of Nitrofen on this day (Kluth et al. 1990; Tenbrinck et al. 1990).

Secondary pulmonary hypoplasia

It is generally assumed in the literature, that lung hypoplasia in cases of CDH is of secondary origin (Gray and Skandalakis 1972; Harrison 1990; Harrison et al. 1980a and 1980b; Wells 1954) and that this hypoplasia is caused by herniated bowel loops compressing the developing fetal lung (Harrison 1990). In contrast to this assumption, our study indicates that pulmonary hypoplasia develops in the embryonic period. Soon after the onset of the defect in the 14-day-old embryo, liver grows through the diaphragmatic defect into the thoracic cavity. This indicates that from this time on the available thoracic space is reduced and further lung growth hampered. In 15- or 16-day-old embryos, the amount of liver inside the chest is only small (Fig. 3A). Therefore, the lungs are nearly normal in these age groups. However, in embryos aged 17 or 18 days, more than one third of available thoracic space may be occupied by liver masses. In these cases, the impaired lung growth is obvious. At first sight, in our embryos pulmonary hypoplasia seems to be the result of compression caused by the ingrowing liver. However, serial section analysis reveals that clear signs of lung compression are absent (Fig. 7). Furthermore, it is obvious (Fig. 8) that in the embryo, the thoracic cavity expands despite the ingrowth of liver. Lung compression is therefore unlikely in a fast growing embryo.

Herniated gut was found in our embryos and fetuses only in late stages of development (21 days and newborns). In all of these, the lungs were already hypoplastic, when the bowel entered the thoracic cavity (Fig. 6B). Based on our observations, we conclude that the early ingrowth of the liver through the diaphragmatic defect is the crucial step in the pathogenesis of pulmonary hypoplasia in CDH. The presence of liver inside the fetal thoracic cavity was recently considered to be of major prognostic importance in the outcome of a fetus with CDH (Harrison et al. 1990). Our pathogenetic concept clearly explains this observation. We found that impaired lung development is proportional to the size of the liver mass inside the thoracic cavity. This indicates that growth impairment is not the result of lung compression in the fetus but rather the result of growth competition

in the embryo: the liver that grows faster than the lung reduces the available thoracic space. If the remaining space is too small, pulmonary hypoplasia will result.

4.6 References

Adzick NS, Harrison MR, Glick PL, Nakayama DK, Manning FA, DeLorimier AA: Diaphragmatic hernia in the fetus: prenatal diagnosis and outcome in 94 cases. J Pediatr Surg 20:357-361, 1985.

Ambrose AM, Larson PS, Borzelleca JF, Smith RB, Hennigar GR: Toxicologic studies on 2,4-dichlorophenyl-p-nitrophenyl ether. Toxicol Appl Pharmacol 19:263-275, 1971.

Broman I: Über die Entwicklung des Zwerchfells beim Menschen. Verh Anat Ges 16:9-17, 1902.

DeLorimier AA, Tierney DF, Parker HR: Hypoplastic lungs in fetal lambs with surgically produced congenital diaphragmatic hernia. Surgery 62:12-17, 1976.

Gray SW, Skandalakis JE: Embryology for surgeons. Philadelphia, PA, Saunders, 1972, pp 359-385.

Harrison MR: The fetus with a diaphragmatic hernia: pathophysiology, natural history, and surgical management. In: Harrison MR, Golbus MS, Filly RA (eds): The unborn patient. Fetal diagnosis and treatment (ed 2). Philadelphia, PA, Saunders, 1990, pp 295-319.

Harrison MR, Jester JA, Ross NA: Correction of congenital diaphragmatic hernia in utero. I. The model: intrathoracic balloon produces fetal pulmonary hypoplasia. Surgery 88:174-182, 1980.

Harrison MR, Bressack MA, Churg AM: Correction of congenital diaphragmatic hernia in utero. II. Simulated correction permits fetal lung growth with survival at birth. Surgery 88:260-268, 1980.

Harrison MR, Langer JC, Adzick NS, Golbus MS, Filly RA, Anderson RL, Rosen MA, Kallen PW, Goldstein RB, DeLorimier AA: Correction of congenital diaphragmatic hernia in utero. V. Initial clinical experience. J Pediatr Surg 25:47-57, 1990.

Iritani I: Experimental study on embryogenesis of congenital diaphragmatic hernia. Anat Embryol 169:133-139, 1984.

Kluth D, Petersen C, Zimmermann HJ, Mühlhaus K: The embryology of congenital diaphragmatic hernia. In: Puri P (ed): Congenital diaphragmatic hernia. Mod Probl Paediatr. Vol 24. Basel, Switzerland, Karger, 1989, pp 7-21.

Kluth D, Kangah R, Reich P, Tenbrinck R, Tibboel D, Lambrecht W: Nitrofen-induced diaphragmatic hernias in rats: an animal model. J Pediatr Surg 25:850-854, 1990.

Nakao Y, Iritani I, Kishimoto H: Experimental animal model of congenital diaphragmatic hernia induced chemically. Teratology 24:11A, 1981.

Nakao Y, Ueki R: Congenital diaphragmatic hernia induced by Nitrofen in mice and rats: characteristics as animal model and pathogenetic relationship between diaphragmatic hernia and lung hypoplasia. Congen Anom 27:397-417, 1987.

Starck D: Embryologie Stuttgart, Germany, Thieme, 1975, pp 488-500.

Tenbrinck R, Tibboel D, Gaillard JLJ, Kluth D, Bos AP, Lachmann B, Molenaar JC: Experimentally induced congenital diaphragmatic hernia in rats. J Pediatr Surg 25:426-429, 1990.

Ueki R, Nakao Y, Nishida T, Nakao Y, Wakabayashi T: Lung hypoplasia in developing mice and rats induced by maternal exposure to Nitrofen. Cong Anom 30:133-143, 1990.

Wells LJ: Development of the human diaphragm and pleural sacs. Contr Embryol Carneg Inst 35:107-137, 1954.

Witschi E: Development of the rat. In: Altman P, Dittmer DS (eds): Growth including reproduction and morphological development. Washington DC, Federation of Societies for Experimental Biology, 1962, pp 304-414.

Chapter 5

PROLIFERATION AND DIFFERENTIATION IN EARLY FETAL RAT LUNGS

A light microscopical and immunohistochemical study in a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia

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PROLIFERATION AND DIFFERENTIATION IN EARLY FETAL RAT LUNGS

A light microscopical and immunohistochemical study in a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia

5.1 Summary

The aim of this study was to investigate the effect of 2,4-dichlorophenyl-p-nitrophenylether (Nitrofen) on early fetal lung development, and to distinguish between effects on proliferation and effects on differentiation of the lung primordium. Pregnant rats were exposed by gavage to Nitrofen (400 mg/kg body weight on day 10 of gestation). Fetuses were collected by Caesarean section on days 11, 12, 13, 15, 17, and 21 (birth on day 23); fetuses or their lungs were embedded in paraffin and sectioned. As criteria for proliferation were taken measurements of length of the pulmonary primordium and staining for PCNA. As differentiation markers we used antibodies against the collagens III and IV, fibronectin, and laminin. Measurements of length showed that the early growth of the lung primordium was reduced on day 13 after exposure to Nitrofen. No differences between normal fetal rat lungs and Nitrofen-exposed fetal rat lungs were found using the other markers.

5.2 Introduction

Lung hypoplasia is an important cause of neonatal mortality in man. It occurs isolated, or in conjunction with other anomalies, such as hydrops fetalis, renal anomalies, hernias (including congenital diaphragmatic hernia and omphalocele), skeletal anomalies, and abnormalities of amniotic fluid, e.g. oligohydramnios and polyhydramnios (Nakamura et al. 1992). In the case of congenital diaphragmatic hernia (CDH), the mortality rate (±50%) mainly depends on the gravity of pulmonary hypoplasia.

To study pulmonary development in CDH we used a rat model: administration of the herbicide 2,4-dichlorophenyl-p-nitrophenyl (Nitrofen) to the mother on day 10 of pregnancy with resulting CDH and pulmonary hypoplasia in 80% of the offspring. Previous studies using this model have shown that Nitrofen-exposed, and thus hypoplastic, late fetal rat lungs are retarded with respect to the differentiation of cuboid type II cells into squamous type I cells whether or not CDH is present, and with respect to the development of the future air spaces between days 20 and 22 if CDH is present (Brandsma

et al. 1994b). However, an obvious effect of this retardation on the composition of extracellular surfactant obtained by bronchoalveolar lavage could not be demonstrated (Brandsma et al. 1993).

A scanning electron microscopical study performed by Kluth et al. (1993) suggests that the early ingrowth of the liver into the thoracic cavity through the diaphragmatic defect may be crucial in the pathogenesis of pulmonary hypoplasia in CDH by reduction of the available thoracic space.

Because of the similarity in stereochemical structure between Nitrofen and thyroid hormones, and the known trophic effect of thyroid hormones on lung development, Manson (1986) hypothesized that Nitrofen exposure has a thyreo*mimetic* effect on the conceptus. This author suggested that this effect would be manifested as a transient stimulation of lung differentiation at the expense of lung growth. The results of our own study concerning the effect of Nitrofen on the binding of thyroid hormone to its receptor (Brandsma et al. 1994c), rather indicate a thyreo*static* challenge to the conceptus resulting in retarded differentiation of alveolar epithelial cells and decreased growth of the lungs.

The aim of the present study was to differentiate between those two theories by investigating the effect of Nitrofen on **early** fetal lung development, and to distinguish between effects on proliferation and effects on differentiation of the lung primordium.

5.3 Materials and Methods

Tissues

Pulmonary hypoplasia in association with CDH was induced as described before (Kluth et al. 1990; Tenbrinck et al. 1990): Specific pathogen free-derived adult rats (Riv:TOX, National Institute of Health and Environmental Protection -RIVM-, Bilthoven, The Netherlands) were mated (gestation day 0). On gestation day 10, a single dose of 400 mg/kg body weight Nitrofen dissolved in olive oil was administered to the dams through an intragastric tube. Pregnant control animals received the same dose of olive oil without Nitrofen. Fetuses were collected by Caesarean section on days 11, 12, 13, 15, 17, and 21 (birth on day 23). In the youngest embryos the number of somites were counted and/or the crown-rump length was measured; of the older fetuses the body weight was determined; they were killed by decapitation. Presence, position and size of a diaphragmatic defect were evaluated if possible. Each litter was randomly subdivided into four groups. The fetuses of one of those groups (53 Nitrofen-exposed fetuses and 55 control fetuses) were used in this study; they were fixed in 2% acetic acid in 100% ethanol, dehydrated in a graded alcohol series, and embedded in paraffin.

Antisera

A monoclonal antibody against Proliferating Cell Nuclear Antigen (PCNA) was purchased from DAKO, Glostrup, Denmark, and was used at a dilution of 1:750. At this dilution, about one third of the crypts in a section of adult rat intestines stained for PCNA.

Affinity-purified goat antibody against type III collagen was obtained from Southern Biotechnology Associates, Inc., Birmingham, Alabama, and was used at a dilution of 1:100.

A monoclonal antibody against type IV collagen (M3F7) was purchased from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD. Anti-collagen IV was used at a dilution of 1:50.

Rabbit anti-fibronectin antiserum was a kind gift of Dr. B. Bachra, Sylvius Laboratory, Leiden, The Netherlands. The serum was used at a dilution of 1:2000.

Rabbit antiserum againts laminin obtained from E-Y Laboratories, Inc., San Mateo, California, was used at a dilution of 1:1000.

Staining procedures

The paraffin-embedded tissue was cut into sections of 5 μm thickness. Some of these sections were stained with hematoxylin and eosin (H&E); these were used for light microscopical investigation of lung growth and architecture of the pulmonary acinus. Adjacent sections were incubated with the above-mentioned antibodies. Prior to incubation the sections were deparaffinated in a graded alcohol series. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in phosphate buffered saline (PBS, pH 7.3); to reduce background staining the antibodies were diluted in PBS containing 0.1% bovine serum albumine. Then the primary antibody was applied to the sections for overnight incubation, followed by incubation with the appropriate peroxidase-labelled secondary antibody for 1½h. After staining with the primary antibodies anti-collagen IV, anti-laminin, and anti-PCNA, and the secondary antibodies, an additional peroxidase anti-peroxidase (PAP, 1:500) was applied also for 1½h to intensify staining results (Sternberger 1986). Each incubation was followed by rinsing with PBS (3x10 min). Subsequently the sections were stained with 0.04% diamino benzidine tetrahydrochloride (DAB) in 0.05M tris maleic acid (pH 7.6) with 0.006% H_2O_2 and either with or without 0.05% $NiCl_2$ for 5-10 min. The reaction was stopped with PBS and the sections were counterstained with methyl green or hematoxylin.

5.4 Results

Proliferation

Hematoxylin and eosin staining: The lung primordium was visible from day 12 of gestation, initially as an outgrowth of the bottom of the foregut, and on day 13 as a triplicate tube growing in caudal direction. The longitudinal growth speed of the lungs exceeded the growth of the whole embryo, resulting on day 15 in a longitudinal lung length of 20% of the embryo (Fig. 1). During this period the lung primordium consisted of tubules lined by (high) columnar primordial epithelial cells surrounded by mesenchymal tissue. The developing diaphragm could be seen from day 14 onward, and was still open on day 15.

Exposure to Nitrofen on day 10 reduced the early growth of the lung primordium, as measured in serial longitudinal sections of exposed embryos on day 13 (Fig. 2). In Nitrofen-exposed fetuses that did not develop CDH, lung length had recovered to control range by day 21 (Fig. 3). In fetuses that did develop a diaphragmatic defect, which always occurred on the left side in this model, smaller left lungs were found in conjunction with right lungs that were significantly longer than control lungs (Fig. 3).

PCNA: Anti-PCNA yields a nuclear staining pattern. On days 11, 12 (Fig. 4), and 13, approximately 100% of the epithelial cells of the foregut and the lung primordium stained for PCNA in both the control group and the Nitrofen-exposed group. On day 15, not all primordial epithelial cells in the lung were positive. Counting of the number of positive nuclei per volume of epithelial cells showed no difference between control lungs and lungs from the Nitrofen-exposed fetuses. On day 17, 95-100% of bronchial and alveolar epithelial cells stained for PCNA, while on day 21 only few positive cells were still seen in both groups.

Also, there were no differences in PCNA-staining of mesenchymal cells between the control group and the Nitrofen-exposed group.

Differentiation

Collagen III: On day 11, no expression of collagen III in the foregut was observed. On day 12, a weak staining for collagen III was found underneath the epithelium of the lungbuds. On day 13, the staining became more intense; a sharp linear pattern surrounded the epithelium of the primordial tubules, and some staining was displayed in the mesenchyme. On days 15, 17 (Fig. 5) and 21, a similar staining pattern was found: a linear pattern around the (primordial) tubules, which could be very faint or very sharp, and in the mesenchyme, especially in the central parts of the lungs, the interlobar septa, and around large bronchi and vessels.

There were no differences found in collagen III expression between control lungs and lungs from Nitrofen-exposed rats.

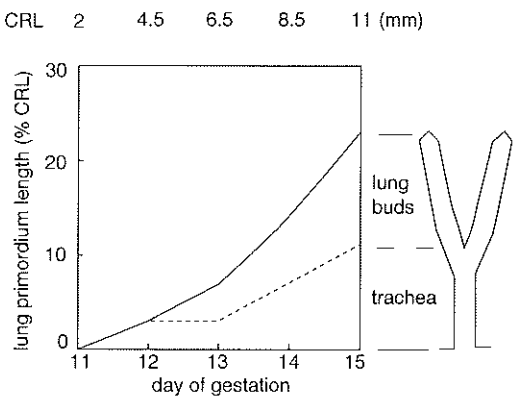


Figure 1 *Length of the lung primordium in relation with crown-rump length (CRL) of control rat embryos between days 11 and 15 of gestation. The growth of the lungs exceeds the growth of the whole embryo.*

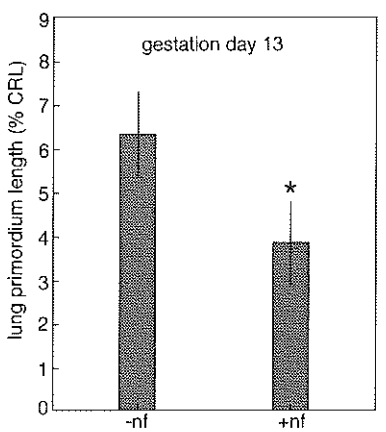


Figure 2 *Significant reduction of lung primordium length on day 13 of gestation in rat embryos after Nitrofen-exposure on day 10 of gestation ($n=4$, $p<0.05$).*

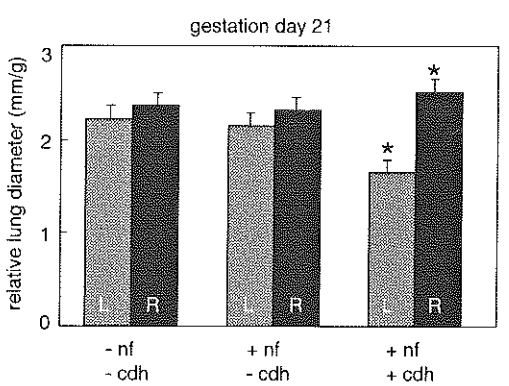


Figure 3 *Longitudinal fetal lung diameter in relation with fetal body weight on day 21 in control and Nitrofen-exposed fetal rats. In the absence of CDH, Nitrofen-exposed fetuses are indistinguishable from controls, whereas in the presence of CDH (always leftsided) left lungs are significantly shorter and right lungs are significantly longer than in control animals ($n=44$, 81, 37; $p<0.05$).*

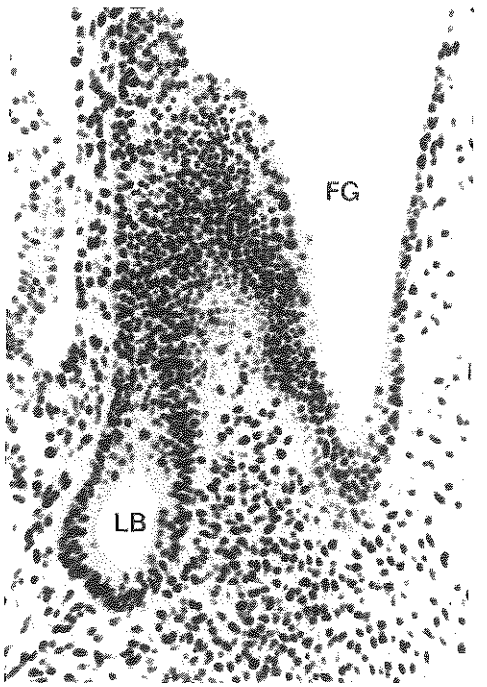


Figure 4 Fetal rat lung bud (LB; day 12), visible as an outgrowth of the foregut (FG), and stained for PCNA. Approximately 100% of the epithelial cells are positive for PCNA. 160x.

Collagen IV: On day 11, the basal lamina and the mesenchyme underneath the foregut epithelium expressed collagen IV. On days 12, 13, 15, 17 (Fig. 6), and 21, staining for collagen IV was found in the basal lamina around (primordial) tubules and blood vessels. Staining intensity varied; around tubules growing towards the pleura staining was often absent on days 15 and 17.
No differences in collagen IV distribution were found between control lungs and lungs from Nitrofen-exposed rats.

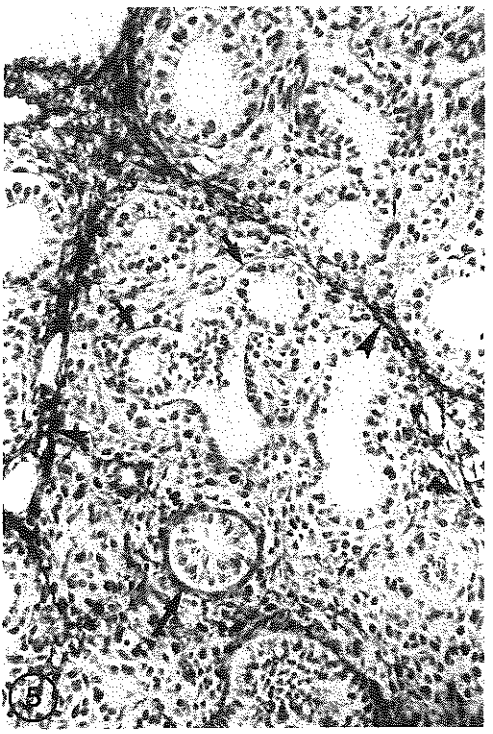
Fibronectin: On day 11, some staining for fibronectin was seen underneath the epithelium of the foregut. On days 12 and 13, staining was seen under the epithelium of the primordial tubules and in endothelial cells. On day 15 (Fig. 7), staining intensity around the tubules varied: underneath high columnar epithelial cells staining was much more intense than under low columnar to high cuboid cells. On day 17, this pattern was even more pronounced: under high columnar bronchial epithelial cells a thick layer of fibronectin was expressed, while under cuboid alveolar epithelial type II cells only a thin, sharp line was seen, or, more peripherally, faint to no staining at all was found. Additionally, endothelial cells did stain and some mesenchymal staining was seen as well. On day 21, only endothelial and some mesenchymal staining was present.
No differences in fibronectin expression were found between control lungs and lungs from Nitrofen-exposed rats.

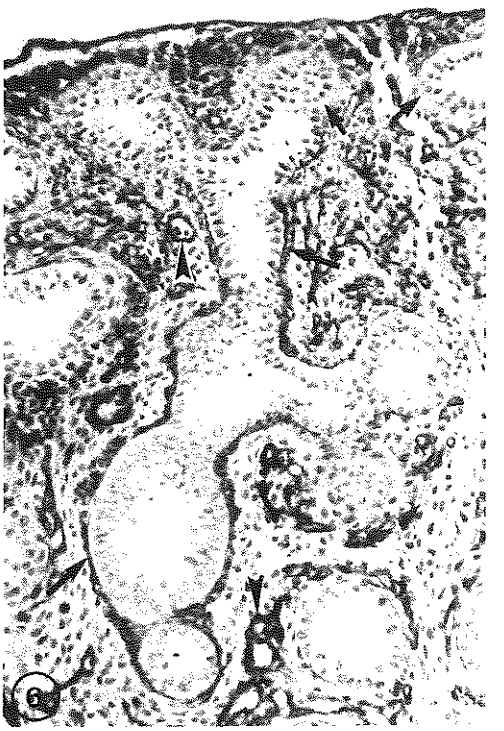
Laminin: On day 11, the foregut epithelium displayed a cytoplasmic staining pattern. On days 12 and 13, the cytoplasm of primordial epithelial cells stained for laminin, and a sharp line was present underneath the epithelial cells. On day 15, a similar staining pattern was displayed, but with less intensity in the high columnar than in the low columnar epithelial cells. Also, endothelial cells stained for laminin. On days 17 (Fig. 8) and 21, no staining was found in columnar bronchial epithelial cells, while cuboid alveolar epithelial cells yielded a cytoplasmic staining pattern.
Again, no differences were found between control lungs and lungs from Nitrofen-exposed rats.

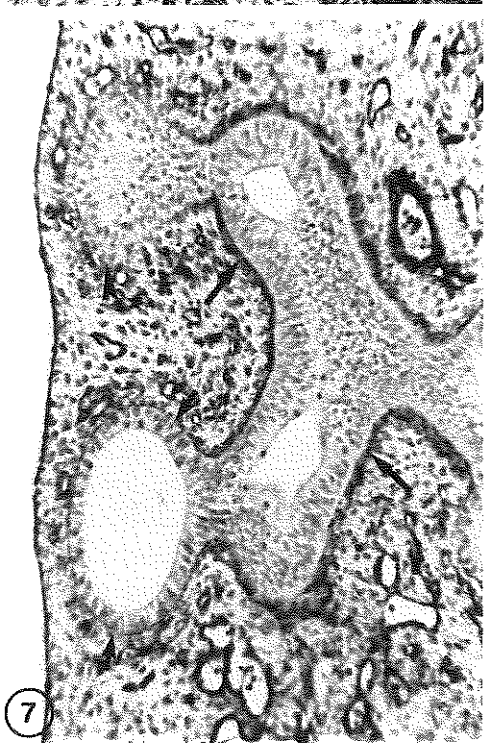
5.5 Discussion

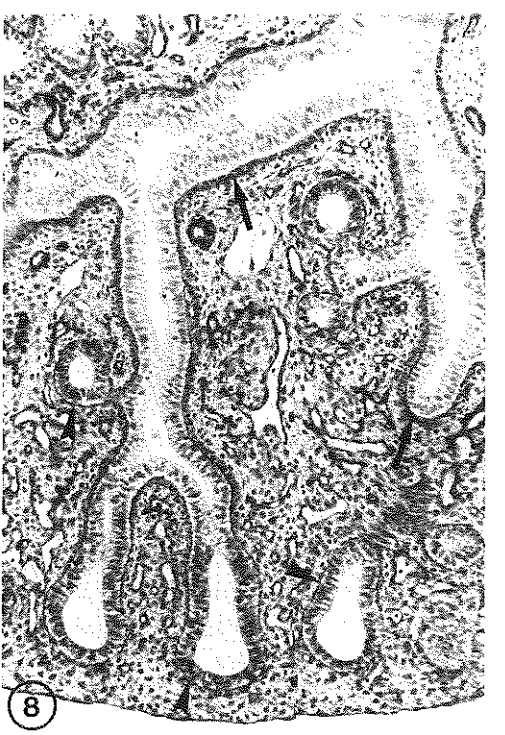
In 10 days, i.e. from day 12 of gestation to term, the rat lung develops from an epithelial protrusion of the foregut into a well differentiated organ with extensive perfusion and diffusion capacities. This requires tissue proliferation as well as differentiation. The epithelial outgrowth proliferates into two lung buds, which, by branching, each develop into a primordial tubular system within a matrix of mesenchyme. The tubules of this primordial system are lined by high columnar primordial epithelial cells on a basal lamina. Further ramification and differentiation leads to the formation of two functionally and morphologically distinctive entities: proximally, the prospective bronchial system and distally, the prospective respiratory system. The bronchial system is lined by high columnar epithelial cells, whereas the respiratory system is initially lined by low columnar or cuboid immature type II cells and later by cuboid type II cells and squamous type I cells (Ten Have-Opbroek 1981). Finally, respiratory tubules, terminal sacs, and alveoli, together with blood capillaries, nervous, and connective tissue will form the adult respiratory system.

Figure 5 *Fetal rat lung (day 17) incubated with anti-collagen III antiserum and stained with a peroxidase-labelled secondary antibody and DAB. A linear staining pattern is present around the acinar tubules (small arrows), around bronchi (arrow) and vessels, and in septa (arrowheads). 200x.*
Figure 6 *Fetal rat lung (day 17) incubated with anti-collagen IV antiserum and stained according to the PAP-technique. A linear staining pattern is present around acinar tubules and bronchi (arrows), and around blood vessels (arrowheads). Note that staining is absent around tubules growing towards the pleura (small arrows). 200x.*
Figure 7 *Fetal rat lung (day 15) incubated with anti-fibronectin antiserum and stained with a peroxidase-labelled secondary antibody and DAB. Staining is present around bronchial and acinar tubules. Underneath high columnar epithelial cells (arrows) staining is much more intense than under low columnar to high cuboid cells (small arrows). Along distalmost acinar tubules no staining is present at all (arrowhead). 200x.*
Figure 8 *Fetal rat lung (day 17) incubated with anti-laminin antiserum and stained according to the PAP-technique. A linear staining is present underneath the epithelial cells of all tubules (arrows). Cuboid alveolar epithelial cells show a cytoplasmic staining for laminin (arrowheads), while columnar bronchial epithelial cells remain negative. 220x.*









Nitrofen exposure results in pulmonary hypoplasia (Tenbrinck et al. 1990; Brandsma et al. 1994b). As mentioned before, it is unknown whether this hypoplasia results from a decreased proliferation or differentiation.

Proliferation

Our findings on day 13, i.e. a decreased length of the lung primordium after Nitrofen-exposure (Brandsma et al. 1994a), and the results from other authors (Iritani 1984; Nakao et al. 1990; Ueki et al 1990) who also describe such an early effect of Nitrofen on lung growth, are not readily explained by Manson's theory (1986). This author suggests a transient stimulation of lung differentiation by Nitrofen at the expense of lung growth. In other words, a stimulation of differentiation precedes, and eventually results in a decreased lung growth. Based on this theory, one would expect to find smaller lungs later in gestation, and not as early as day 13. However, an early effect is of course not excluded by Manson's theory. On the other hand, our before-mentioned findings do suggest a decrease in proliferation rate of the *early* fetal pulmonary epithelium in the Nitrofen-exposed group which may result in a retarded differentiation. Using antibodies against PCNA however, there is no difference in proliferation rate demonstrable between the two experimental groups. This apparent contradiction can be explained in several ways.

Possibly, the onset of the outgrowth of the lung bud starts later, so that the absolute number of proliferating cells at any given moment is smaller than in the control group. Nakao et al. (1990) describe a 6-12 hours delay in lung bud formation in both rat and mice after Nitrofen-exposure compared to control animals. In the other above-mentioned publications (Iritani 1984; Ueki et al. 1990), as in our own work, a decreased lung length or lung weight at an early embryonic age is described, but the onset of lung bud formation is not included in the studies. To investigate this, smaller time intervals between the sacrifices of the experimental animals as well as counting of the total amount of proliferating cells at each time point are necessary.

Another option is that a decreased growth in length of the lung primordium goes along with premature branching, which would not result in a decreased proliferation rate either. A study of the three-dimensional growth of the primordial system could give an answer to this question.

Another explanation for the PCNA staining results is that the method is not suitable for this purpose. The 36 kDa proliferation cell nuclear antigen (PCNA, cyclin) is an intranuclear polypeptide which begins to accumulate during the G1 phase of the cell cycle, is most abundant during the S phase of the cell cycle, and declines during the G2/M phase (Hall and Woods 1990). Therefore, PCNA is a very sensitive detection method for ongoing or recent cell replication, and maybe not so useful in embryonic tissue, in which proliferation rates are expected to be very high. Bromodeoxyuridine (BRDU) incorporation occurs only during S phase, resulting in a lower percentage of positive,

proliferating cells (Zeymer et al. 1992). Use of this method might reveal a difference in proliferation rate between control lungs and Nitrofen-exposed lungs after all. This is currently under study in our laboratory.

Differentiation

Epithelial branching is one of the major events in lung morphogenesis. The branching proces depends on the interaction of the epithelium with the mesenchyme; the amount of mesenchyme as well as its source (lung versus non-lung, terminal versus proximal) is shown to be of importance in in vitro studies (Gross 1990; Hilfer et al. 1985; Masters 1976; Taderera 1967). It seems likely that mesenchyme supports the morphogenesis and differentiation of the respiratory epithelium in part through the type of matrix that it synthesizes and deposits (Brody 1985; Edelson et al. 1989; Lwebuga-Mukasa 1991; McGowan 1992; Rannels and Rannels 1989a,b; Shannon et al. 1987; Warburton et al. 1993). Among the components of the extracellular matrix that have been indicated as being necessary for branching are the collagens (Arden et al. 1993; Chen and Little 1987; Hashimoto and Hoshino 1988; Heine et al. 1990; Nerlich et al. 1985), fibronectin (Chen et al. 1986; Roman and McDonald 1992; Rosenkrans et al. 1983; Snyder et al. 1987), and laminin (Chen et al. 1986; Schuger et al. 1990, 1992). The present study describes the in vivo expression of these extracellular matrix factors in normal and in Nitrofen-exposed, and thus hypoplastic fetal rat lungs. The expression patterns found, correspond with those described by other authors for the collagens (Arden et al. 1993; Brody 1985; Chen and Little 1987; Heine et al. 1990; Nerlich et al. 1985), and fibronectin (Chen et al. 1986; Heine et al. 1990; Roman and McDonald 1992). For laminin we find a different staining pattern than most other authors: in addition to basal lamina staining, we also find staining of primordial epithelial cells and cuboid alveolar type II cells. Schuger et al. (1992) describe that both lung epithelium and mesenchyme produce complete laminin molecules, shown by immunohistochemistry and in situ hybridization studies. Our results do not show any differences in expression pattern of the before-mentioned differentiation markers between control and Nitrofen-exposed rats. Neither Manson's theory of a transient stimulation of differentiation by Nitrofen, nor our own theory of a retarded differentiation after Nitrofen exposure is supported by this outcome. It could be that, though the **pattern** of expression is the same, the quantity of expressed matrix factors differs between the two groups. However, such conclusions can not be drawn from our immunohistochemical results; for this, biochemical analysis would be required.

It is possible that a difference between control and Nitrofen-exposed lungs will be found by staining for other factors that are known to regulate morphogenesis and differentiation of the lung, such as the group of cell adhesion molecules (e.g. integrins, cadherins) or growth factors (e.g. PDGF, TGFß). In a previous study we have shown that Nitrofen reduces the binding of triiodothyronine (T3) to its receptor (Brandsma et al. 1994c). As thyroid hormones are known to have a trophic effect on lung development and

differentiation of epithelial cells, it is of interest to study the expression of the nuclear T3-receptor in early fetal rat lungs exposed to Nitrofen (currently under investigation in our laboratory).

In conclusion, our results from the present study, in which we use a number of proliferation and differentiation markers besides measurements of length in normal and in Nitrofen-exposed lungs, unfortunately do not fully answer the question, whether pulmonary hypoplasia results from a decreased proliferation or differentiation. However, studies by us and other investigators suggest that Nitrofen may possibly cause a retarded onset of lung bud development or an altered growth pattern of the lung such as premature branching, both of which may affect the growth in length of the organ.

5.6 Acknowledgments

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5.7 References

Arden MG, Spearman MA, Adamson IYR: Degradation of type-IV collagen during the development of fetal rat lung. Am J Respir Cell Mol Biol 9:99-105, 1993.

Brandsma AE, Tibboel D, Vulto IM, Egberts J, ten, Have-Opbroek AAW: Ultrastructural features of alveolar epithelial cells in the late fetal pulmonary acinus. A comparison between normal and hypoplastic lungs, using a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia. In: Microscopic evaluation of respiratory tract function. Ten Have-Opbroek AAW and Plopper CG, Eds. Microsc Res Techn 26:389-399, 1993.

Brandsma AE, Piersma AH, Hofstee-Hooftman MWA, Verhoef A, Ten Have-Opbroek AAW, Tibboel D: Early lung development in the rat and its perturbation by Nitrofen exposure. Pediatr Res 36:47A, 1994a.

Brandsma AE, Ten Have-Opbroek AAW, Vulto IM, Molenaar, J.C., Tibboel D: Alveolar epithelial composition and architecture of the late fetal pulmonary acinus: an immunocytochemical and morphometric study in a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia. Exp Lung Res 20:491-515, 1994b.

Brandsma AE, Tibboel D, Vulto I, de Vijlder JJM, ten, Have-Opbroek AAW, Wiersinga WM: Inhibition of T3-receptor binding by Nitrofen. Biochim Biophys Acta 1201:266-270, 1994c.

Brody JS: Cell-to-cell interactions in lung development. Pediatr Pulmonol 1:S42-S48, 1985.

Chen WT, Chen JM, Mueller SC: Coupled expression and colocalization of 140K cell adhesion molecules, fibronectin, and laminin during morphogenesis and cytodifferentiation of chick lung cells. J Cell Biol 103:1073-1090, 1986.

Chen JM, Little CD: Cellular events associated with lung branching morphogenesis including the deposition of collagen type IV. Dev Biol 120:311-321, 1987.

Edelson JD, Shannon JM, Mason RJ: Effects of two extracellular matrices on morphologic and biochemical properties of human type II cells in vitro. Am Rev Respir Dis 140:1398-1404, 1989.

Gross I: Regulation of fetal lung maturation. Am J Physiol 259:L337-L344, 1990.

Hall PA, Woods AL: Immunohistochemical markers of cellular proliferation: achievements, problems and prospects. Cell Tissue Kinet 23:505-522, 1990.

Hashimoto H, Hoshino K: Immunohistochemical localization of laminin and type IV collagen during morphogenesis of fetal lungs in mice. J Histochem Cytochem 36:865, 1988.

Heine UI, Munoz EF, Flanders KC, Roberts AB, Sporn, M.B.: Colocalization of TGF-beta 1 and collagen I and III, fibronectin and glycosaminoglycans during lung branching morphogenesis. Development 109:29-36, 1990.

Hilfer SR, Rayner RM, Brown JW: Mesenchymal control of branching pattern in the fetal mouse lung. Tissue Cell 17:523-538, 1985.

Iritani I: Experimental study on embryogenesis of congenital diaphragmatic hernia. Anat Embryol 169:133-139, 1984.

Kluth D, Kangah R, Reich P, Tenbrinck R, Tibboel D, Lambrecht W: Nitrofen-induced diaphragmatic hernias in rats: an animal model. J Pediatr Surg 25:850-854, 1990.

Kluth D, Tenbrinck R, von Ekesparre M, Kangah R, Reich P, Brandsma A, Tibboel D, Lambrecht W: The natural history of congenital diaphragmatic hernia and pulmonary hypoplasia in the embryo. J Pediatr Surg 28:456-463, 1993.

Lwebuga-Mukasa JS: Matrix-driven pneumocyte differentiation. Am Rev Respir Dis 144:452-457, 1991.

Manson JM: Mechanism of Nitrofen teratogenesis. Environ Health Persp 70:137-147, 1986.

Masters JR: Epithelial-mesenchymal interaction during lung development: the effect of mesenchymal mass. Dev Biol 51:98-108, 1976.

Mcgowan SE: Extracellular Matrix and the Regulation of Lung Development and Repair. FASEB J 6:2895-2904, 1992.

Nakamura Y, Harada K, Yamamoto I, Uemura Y, Okamoto K, Fukuda S, Hashimoto T: Human Pulmonary Hypoplasia - Statistical, Morphological, Morphometric, and Biochemical Study. Arch Pathol Lab Med 116:635-642, 1992.

Nakao Y, Ueki R, Nakao Y, Nishida T, Nomura M, Takahashi, T., Nakao BMSR: Effects of maternal nitrofen exposure on lung organogenesis in mice and rats. Teratology 43A, 1990.

Nerlich AG, Wiestner M, Muller PK: Immunohistological analysis of collagen types in human lung. Fortschr Zool 30:393-395, 1985.

Rannels DE, Rannels SR: Influence of the extracellular matrix on type 2 cell differentiation. Chest 96:165-173, 1989a.

Rannels SR, Rannels DE: The type II pneumocyte as a model of lung cell interaction with the extracellular matrix. J Mol Cell Cardiol 21 Suppl 1:151-159, 1989b.

Roman J, Mcdonald JA: Expression of Fibronectin, the Integrin alpha5, and alpha- Smooth Muscle Actin in Heart and Lung Development. Am J Resp Cell Mol Biol 6:472-480, 1992.

Rosenkrans WA Jr, Albright JT, Hausman RE, Penney DP: Light-microscopic immunocytochemical localization of fibronectin in the developing rat lung. Cell Tissue Res 233:113-123, 1983.

Schuger L, O'Shea S, Rheinheimer J, Varani J: Laminin in lung development: effects of anti-laminin antibody in murine lung morphogenesis. Dev Biol 137:26-32, 1990.

Schuger L, Varani J, Killen PD, Skubitz APN, Gilbride K: Laminin Expression in the Mouse Lung Increases with Development and Stimulates Spontaneous Organotypic Rearrangement of Mixed Lung Cells. Dev Dynam 195:43-54, 1992.

Shannon JM, Emrie PA, Fisher JH, Kuroki Y, Jennings, S.D., Mason RJ: Effect of a reconstituted basement membrane on expression of surfactant apoproteins in cultured adult rat alveolar type II cells. Am J Respir Cell Mol Biol 2:183-192, 1990.

Snyder JM, O'Brien JA, Rodgers HF: Localization and accumulation of fibronectin in rabbit fetal lung tissue. Differentiation 34:32-39, 1987.

Sternberger LA: Immunohistochemistry. John Wiley & Sons, New York. 1986, pp 90-209.

Taderera JV: Control of lung differentiation in vitro. Dev Biol 16:489-512, 1967.

Tenbrinck R, Tibboel D, Gaillard JL, Kluth D, Bos AP, Lachmann B, Molenaar JC: Experimentally induced congenital diaphragmatic hernia in rats. J Pediatr Surg 25:426-429, 1990.

Ten Have-Opbroek AA: The development of the lung in mammals: an analysis of concepts and findings. Am J Anat 162:201-219, 1981.

Ueki R, Nakao Y, Nishida T, Nakao Y, Wakabayashi T: Lung hypoplasia in developing mice and rats induced by maternal exposure to Nitrofen. Cong Anom 30:133-143, 1990.

Warburton D, Lee M, Berberich MA, Bernfield M: Molecular Embryology and the Study of Lung Development. Am J Respir Cell Mol Biol 9:5-9, 1993.

Zeymer U, Fishbein MC, Forrester JS, Cercek B: Proliferating Cell Nuclear Antigen immunohistochemistry in rat aorta after balloon denudation. Comparison with thymidine and bromodeoxyuridine labeling. Am J Pathol 141:685-690, 1992.

Chapter 6

INHIBITION OF T_3-RECEPTOR BINDING BY NITROFEN

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INHIBITION OF T_3-RECEPTOR BINDING BY NITROFEN

6.1 Summary

Lung development is controlled by various hormones, including thyroid hormone. The herbicide 2,4-dichlorophenyl-*p*-nitrophenyl ether (Nitrofen) induces lung hypoplasia in fetal rats, when administered to the mother during gestation. Nitrofen might be teratogenic by an anti-thyroid activity. The present study shows that Nitrofen decreases the binding of T_3 to the α_1 and β_1 form of the thyroid hormone receptor in a non-competitive way. Consequently, rat lung hypoplasia might result from the decreased binding of T_3 to its receptor, via exposure to Nitrofen during fetal development.

6.2 Introduction

Lung development is controlled by various hormones, including glucocorticoids and thyroid hormone. Thyroid hormone is known to have a trophic effect on lung development (Ballard 1983; Gross 1990; Hitchcock 1979). The herbicide 2,4-dichlorophenyl-*p*-nitrophenyl ether (Nitrofen) is a potent teratogen in rodents. Administration of 100 mg Nitrofen to pregnant rats on day 10 of gestation results in pulmonary hypoplasia and a closing defect of the diaphragm known as congenital diaphragmatic hernia in the offspring (Kluth 1990; Tenbrinck 1990). However, exposure of pregnant rats to Nitrofen on other gestational days leads to hydronephrosis, cardiac malformations, and eye anomalies in the neonates (Burke Hurt et al. 1983; Costlow and Manson 1981; Gray et al. 1982; Lau et al. 1986; Ostby et al. 1985). The similarity in stereochemical structure between Nitrofen and thyroid hormones suggested an alteration of thyroid function by Nitrofen (Gray and Kavlock 1983). Exposure of euthyroid pregnant rats to Nitrofen indeed resulted in an initial depression of serum thyroxine (T_4) levels followed by an increase exceeding normal serum T_4 levels in the mother rats at term; serum triiodothyronine (T_3) was normal or slightly elevated (Manson 1986; Manson et al. 1984). In the near term fetus a decreased T_4 level and normal T_3 and thyroid stimulating hormone levels were found (Manson 1986; Manson et al. 1984). After coadministration of T_4 and Nitrofen to pregnant rats a dramatic reduction (70%) in the frequency of malformations in the offspring occurred (Manson et al. 1984). Nitrofen competed with [^{125}I]T_4 for binding to rat thyroid binding globulin in vitro (Manson 1986). From these studies it is likely that Nitrofen exerts its teratogenic effects via alterations in the thyroid hormone metabolism. We investigated the effect of

Nitrofen on the binding of thyroid hormone to the α_1 and $ß_1$ form of the thyroid hormone receptor obtained from bacteria by recombinant DNA techniques. Our results have led to the hypothesis that Nitrofen directly interacts with the nuclear thyroid hormone receptor. In the embryo this may lead to abnormal organogenesis. Some of the observations have been published in an abstract (Brandsma et al. 1993a).

6.3 **Materials and Methods**

Chemicals

Triiodothyronine (T_3) was obtained from Henning GmbH (Berlin, FRG) and [^{125}I]T_3 (specific activity 2200 Ci/mmol) was purchased from New England Nuclear (Boston, USA). 2,4-Dichlorophenyl-*p*-nitrophenyl ether (Nitrofen) was a generous gift from Rohm and Haas Company (Springhouse, Philadelphia).

Receptor proteins

The pEX plasmid (Stanley and Luzio 1984) was used as a bacterial expression vector in the E.coli pop 2136 strain (Vidal-Ingigliardi and Raibaud 1985) containing the cIts857 repressor. The c-ERB A coding sequences of chicken α type (full length, encoding amino-acids 1-408) (Sap et al. 1986), or rat ß type (encoding amino acids 31-456) (Murray et al. 1988) were introduced into this vector. Both sequences contain the hormone- and DNA binding domain. In addition, the affinity constants (K_a) of the receptor proteins for T_3 ($K_a = 1.1\ 10^{10}$ for α_1; $K_a = 5.2\ 10^9$ for $ß_1$) are in the same range as we found for whole nuclei ($K_a = 2.2\ 10^9$) and as described in the literature (Van der Klis et al. 1991, Wiersinga 1985). C-ERB A fusion proteins were isolated as described by Stanley and Luzio (1984) and stored in solution A (20 mM Tris-HCl, 0.25 M sucrose, 1 mM EDTA, 50 mM NaCl, 5% (v/v) glycerol, pH 7.6) in liquid N_2. Polyacrylamide-gelelectroforesis of the proteins after incubation with Bromo-acetyl-[^{125}I]T_3 showed that approximately 10 to 15% of total protein expressed by the bacterial system is the α_1 or $ß_1$ receptor (Van der Klis et al. 1991). The molecular weight of the receptor proteins was calculated as 45 to 50 kD. Autoradiography of the gel demonstrated that only proteins with a Mw corresponding to the receptor bind T_3 (Van der Klis et al. 1991).

Binding studies

Prior to each incubation the fusion protein suspension was thawed, solubilized by sonification (10 seconds, 6 microns), and the non-soluble fraction was pelleted by centrifugation (10,000xg, 5 minutes, 4°C). 40 μl c-ERB A protein suspension was added to solution A with [^{125}I]T_3 and 5 mM dithiothreitol in the absence or presence of various concentrations of nonradioactive T_3 and/or Nitrofen dissolved in 7.5% ethanol. 500 μl of this mixture was incubated for 2 hours at 22°C in a shaking water bath. Incubations were

stopped by chilling the samples in ice-cold water. Separation of bound and unbound hormone was performed at 4°C by elution with solution A using Sephadex G-25 Medium (Pharmacia, Uppsula, Sweden) columns in a Pasteur pipette (bed volume approximately 2.5 ml) (Hartong and Wiersinga 1985). Fractions of 0.8 ml were collected from the columns and the ^{125}I radioactivity measured. The first four fractions were taken to represent the hormonal fraction bound to the c-ERB A proteins (Hartong and Wiersinga 1985). Specific binding was determined by subtracting the non-specific binding, i.e. $[^{125}I]T_3$ bound to c-ERB A protein in the presence of an excess (10^{-7}M) nonradioactive T_3.

6.4 Results

Mean specific binding of $[^{125}I]T_3$ to the expressed α_1 chicken and $ß_1$ rat receptor proteins under the conditions used was 44.9% and 31.5% respectively; mean non-specific binding was 4.3% and 4.6%. Expression products of the vector pEX without c-ERB A coding sequences did not bind $[^{125}I]T_3$ specifically. A linear relationship existed between specific binding and protein concentration for both the α_1 and $ß_1$ type of thyroid hormone receptor up to 45 μl protein suspension.
Incubation of the receptor proteins with $[^{125}I]T_3$ in the presence of the solvent ethanol (7.5%) reduced the mean specific binding to 40.0% and 19.8% for α_1 and $ß_1$ respectively. Incubation in the presence of 1mM Nitrofen further reduced the mean specific binding to 30.5% and 14.8%. Reduction of T_3-binding to the α_1 receptor in the presence of 100 μM Nitrofen is shown in figure 1.

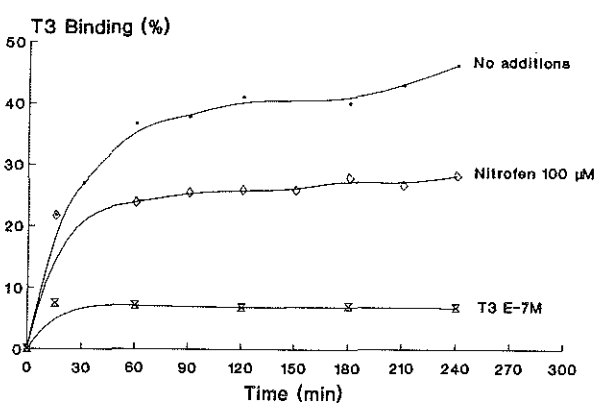

Figure 1 Binding of $[^{125}I]T_3$ to the α_1 receptor. The α_1 receptor was added to solution A with $[^{125}I]T_3$ in the absence or presence of Nitrofen or an excess of nonradioactive T_3, and incubated at 22°C. At various time points a sample was taken, bound $[^{125}I]T_3$ was separated and counted. Note that both Nitrofen and an excess of nonradioactive T_3 reduce the binding of $[^{125}I]T_3$ to the receptor.

Scatchard analysis showed that ethanol decreased the affinity (slope of the curve) of T3 for the receptor, whereas Nitrofen decreased the maximal binding capacity (MBC; intercept on the x-axis) of the receptor for T3 in both the α_1 (Fig. 2) and $ß_1$ (not shown) receptor type. The affinity constant K_a of T_3 for the α_1 T_3-receptor in the presence of

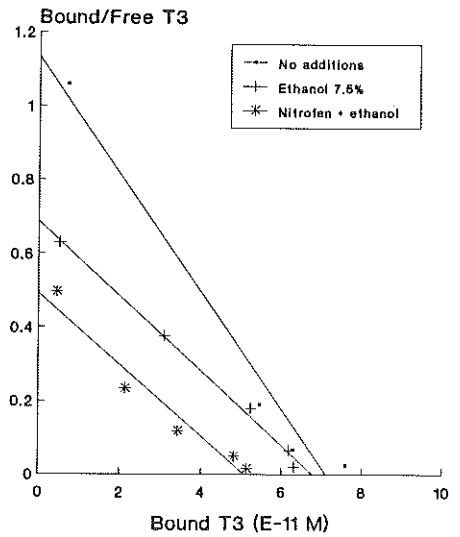

Figure 2 *Scatchard plots of the binding of T_3 to the α_1 receptor. The α_1 receptor was added to solution A with $[^{125}I]T_3$ in the absence or presence of Nitrofen or 7.5% ethanol, and in the presence of various concentrations nonradioactive T_3. After 2 hours incubation at 22°C the amount of bound T_3 was counted and Scatchard plots were made: without any additions (K_a $1.58 \cdot 10^{10}$L/M; MBC $7.11 \cdot 10^{-11}$M), in the presence of 7.5% ethanol (K_a $1.01 \cdot 10^{10}$L/M; MBC $6.8 \cdot 10^{-11}$M), and in the presence of 1mM Nitrofen dissolved into 7.5% ethanol (K_a $0.94 \cdot 10^{10}$L/M; MBC $5.16 \cdot 10^{-11}$M). It is shown that ethanol decreases the affinity constant (K_a), whereas Nitrofen decreases the maximal binding capacity (MBC).*

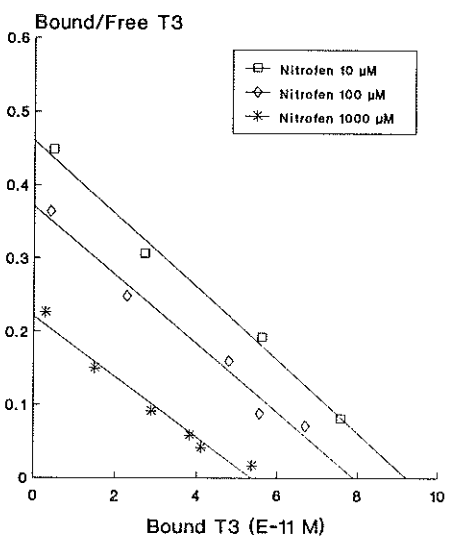

Figure 3 *Scatchard plots of the binding of T_3 to the α_1 receptor. The α_1 receptor was added to solution A with $[^{125}I]T_3$ in the presence of various concentrations of Nitrofen and nonradioactive T_3. After 2 hours incubation at 22°C the amount of bound T_3 was counted and Scatchard plots were made. Note the dose dependent decrease of maximal binding capacity by Nitrofen.*

Nitrofen and the solvent ethanol was $0.6\ 10^{10} \pm 0.18\ 10^{10}$ L/M. The MBC in the presence of ethanol only was $7.6\ 10^{-11} \pm 0.84\ 10^{-11}$ M, whereas the MBC in the presence of 1 mM Nitrofen and ethanol was $4.96\ 10^{-11} \pm 0.99\ 10^{-11}$ M ($p=0.04$). The effect of Nitrofen was dose-dependent (Fig. 3).

Lineweaver-Burk analysis of the experiments depicted in figure 1 and 2 revealed a non-competitive inhibition by Nitrofen, as shown by intersection of the lines on the base line (Figs. 4, 5). In accordance with the Scatchard analysis, the solvent ethanol appeared to be a competitive inhibitor (Fig. 4). The inhibition constant K_i for Nitrofen could be

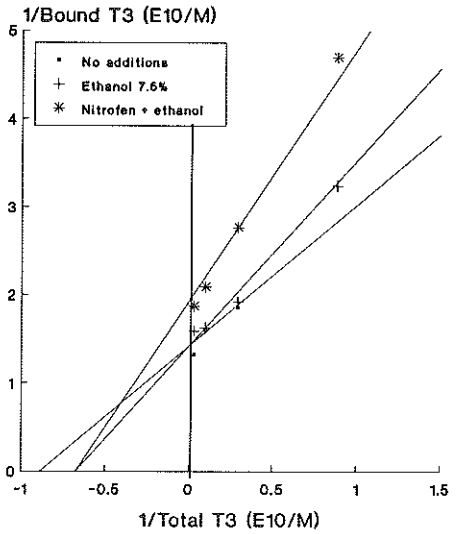

Figure 4 Lineweaver-Burk plots of the effect of 7.5% ethanol and 1mM Nitrofen dissolved into 7.5% ethanol on the binding of T_3 to the α_1 receptor type. The α_1 receptor was added to solution A with $[^{125}I]T_3$ in the absence or presence of Nitrofen or 7.5% ethanol, and in the presence of various concentrations nonradioactive T_3. After 2 hours incubation at 22°C the amount of bound T_3 was counted and Lineweaver-Burk plots were made. The 'ethanol line' and the 'Nitrofen line' intersect on the base line, revealing a non-competitive inhibition mechanism, while the 'no addition and ethanol lines' intersect at the y-axis, showing that ethanol is a competitive inhibitor.

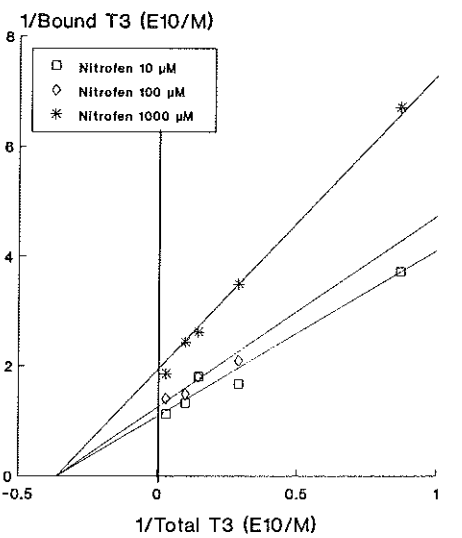

Figure 5 Lineweaver-Burk plots of the effect of Nitrofen on the binding of T_3 to the α_1 receptor type. The α_1 receptor was added to solution A with $[^{125}I]T_3$ in the presence of various concentrations of Nitrofen and nonradioactive T_3. After 2 hours incubation at 22°C the amount of bound T_3 was counted and Lineweaver-Burk plots were made. Note the intersection of all three lines on the baseline, revealing a non-competitive inhibition mechanism.

determined graphically by plotting 1/Bound T_3 versus the concentration of Nitrofen. For non-competitive inhibitors the intercept of the x-axis represents the K_i-value. This was found to be 1.2 10^{-3}M for the α_1 protein (Fig. 6) and 10^{-3}M for the $ß_1$ protein (not shown). Delayed addition of Nitrofen to the incubation mixture resulted in a decrease of $[^{125}I]$T3 binding in a similar way as delayed addition of non-radioactive T3; the two effects seemed to be additive (Fig. 7).

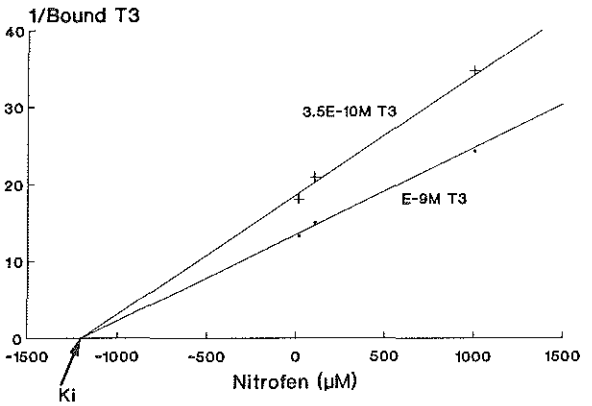

Figure 6 *K_i plot: graphical determination of the inhibitor constant K_i of Nitrofen for the binding of T_3 to the α_1 receptor type.*

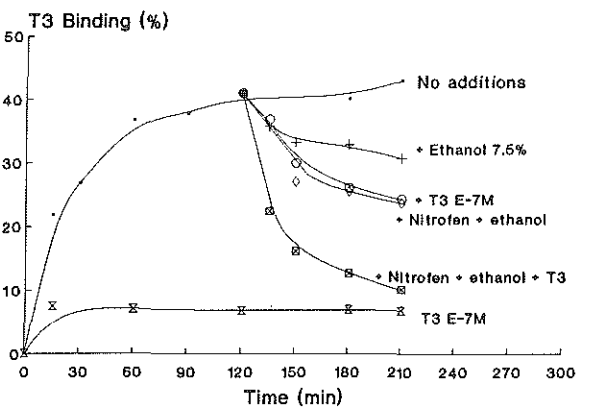

Figure 7 *Binding of $[^{125}I]T_3$ to the α_1 receptor. The α_1 receptor was added to solution A with $[^{125}I]T_3$ in the absence or presence of an excess of nonradioactive T_3, and incubated at 22°C. After 2 hours 7.5% ethanol, Nitrofen, nonradioactive T_3, or a combination of Nitrofen and T_3 was added to the incubation mixture. At various time points a sample was taken, and bound $[^{125}I]T_3$ was separated and counted. Dissociation of bound T_3 occurred by the addition of Nitrofen, nonradioactive T_3, or a combination of the two.*

6.5 **Discussion**

Our results demonstrate that Nitrofen inhibits the binding of $[^{125}I]T_3$ to the α_1 and β_1 form of the recombinant thyroid hormone receptor. The inhibition is dose dependent and non-competitive in character. Rather high concentrations of Nitrofen are necessary to achieve the inhibitory effect. Nitrofen is known to be a very lipophilic substance; concentrations of Nitrofen in fat can easily be 100 times higher than in blood (Brown and Manson 1986; Costlow and Manson 1983). In accordance with the accumulation in fat, we think that Nitrofen can also concentrate in lipoid membranes, and thus locally reach very high concentrations. If Nitrofen binds to the same binding site as T_3, which has been suggested by Manson (1986) based on the resemblance in stereochemical configuration, the inhibition mechanism would be competitive in character. The observed non-competitive inhibition indicates that Nitrofen binds to a different site on the receptor than T_3 itself.

In addition to the inhibitory effect of Nitrofen on the binding of T_3 to the T_3-receptor, maternal and fetal serum T_4 levels *de*crease after Nitrofen-exposure. Also, lungs become hypoplastic after Nitrofen-exposure (Tenbrinck et al. 1990), and a retarded development of the future air spaces (Brandsma et al. 1992), of type I (Brandsma et al. 1992) and of type II alveolar epithelial cells (Brandsma et al. 1993b) occurs. All these findings are not in agreement with a thyreomimetic effect, as suggested by Manson (1986). This author states that Nitrofen exposure results in a thyreomimetic challenge to the conceptus, because slight increases in phospholipid levels were measured and a dose-related increase in malic enzyme activity was found late in gestation. However, from our findings we must conclude that Nitrofen-exposure most likely results in a thyreostatic challenge to the conceptus. This effect can be achieved by a non-competitive inhibition of the binding of T_3 to the nuclear T_3-receptor by Nitrofen.
The expression of the nuclear T_3-receptor in fetal rat lungs and the distribution of Nitrofen in the fetus are currently under investigation in in vivo experiments. Nitrofen might possibly interfere also at other levels with thyroid hormone metabolism.

6.6 Acknowledgments

We thank Dr. E.D.L. Schmidt for advising us and for making the E-coli containing the c-ERB A gen available to us, and Dr. F.R.M. van der Klis for her practical advices and assistance.

6.7 References

Ballard PL: In Abnormal Functional Development of the Heart, Lungs, and Kidneys: Approaches to Functional Teratology, Alan R Liss, Inc, New York, NY, 1983, pp 103-117.

Brandsma AE, Ten Have-Opbroek AAW, Vulto IM, Molenaar JC, Tibboel D: Alveolar epithelial composition and architecture in a rat model of lung hypoplasia. Am Rev Resp Dis 145(Suppl):A126, 1992.

Brandsma AE, Tibboel D, Vulto IM, Ten Have-Opbroek AAW, Wiersinga WM: Lung hypoplasia is induced by Nitrofen: an effect via inhibition of T_3-receptor binding? Am Rev Resp Dis 147(Suppl):A415, 1993a.

Brandsma AE, Tibboel D, Vulto IM, Egberts J, Ten Have-Opbroek AAW: Ultrastructural features of alveolar epithelial cells in the late fetal pulmonary acinus: a comparison between normal and hypoplastic lungs using a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia. Microsc Res Techn 26:389-399, 1993b.

Brown TJ, Manson JM: Further characterization of the distribution and metabolism of Nitrofen in the pregnant rat. Teratology 34:129-139, 1986.

Burke Hurt SS, Smith JM, Hayes AW: Nitrofen: a review and perspective. Toxicology 29:1-37, 1983.

Costlow RD, Manson JM: The heart and diaphragm: Target organs in the neonatal death induced by Nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether). Toxicology 20:209-227, 1981.

Costlow RD, Manson JM: Distribution and metabolism of the teratogen Nitrofen (2,4-dichloro-4'-nitro diphenyl ether) in pregnant rats. Toxicology 26:11-23, 1983.

Gray LE, Kavlock RJ: The effects of the herbicide 2,4-dichlorophenyl-p-nitrophenyl ether (Nit) on serum thyroid hormones in adult female mice. Toxicol Lett 15:231-235, 1983.

Gross I: Regulation of fetal lung maturation. Am J Physiol 259:L337-L344, 1990.

Hartong R, Wiersinga WM: Nuclear reverse T3 binding sites: an artefact of isolation? Acta Endocrinol (Copenh) 108:525-531, 1985.

Hitchcock KR: Hormones and the lung. I. Thyroid hormones and glucocorticoids in lung development. Anat Rec 194:15-39, 1979.

Kluth D, Kangah R, Reich P, Tenbrinck R, Tibboel D, Lambrecht W: Nitrofen-induced diaphragmatic hernias in rats: an animal model. J Pediatr Surg 25:850-854, 1990.

Lau C, Cameron AM, Irsula O, Robinson KS: Effects of prenatal Nitrofen exposure on cardiac structure and function in the rat. Toxicol Appl Pharmacol 86:22-32, 1986.

Manson JM: Mechanism of Nitrofen teratogenesis. Environ Health Persp 70:137-147, 1986.

Manson JM, Brown T, Baldwin DM: Teratogenicity of Nitrofen (2,4,-dichloro-4'-nitrodiphenyl ether) and its effects on thyroid function in the rat. Toxicol Appl Pharmacol 73:323-335, 1984.

Murray MB, Zilz ND, McCreary NL, MacDonald MJ, Towle HC: Isolation and characterization of rat cDNA clones for two distinct thyroid hormone receptors. J Biol Chem 263:12770-12777, 1988.

Ostby JS, Gray LE, Kavlock RJ, Ferrell JM: The postnatal effects of prenatal exposure to low doses of Nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) in Sprague-Dawley rats. Toxicology 34:285-297, 1985.

Sap J, Munoz A, Damm K, Goldberg Y, Ghysdael J, Leutz A, Beug H, Vennstrom B: The c-erb-A protein is a high-affinity receptor for thyroid hormone. Nature 324:635-640, 1986.

Stanley KK, Luzio JP: Construction of a new family of high efficiency bacterial expression vectors: identification of cDNA clones coding for human liver proteins. EMBO J 3:1429-1434, 1984.

Tenbrinck R, Tibboel D, Gaillard JLJ, Kluth D, Bos AP, Lachmann B, Molenaar JC: Experimentally induced congenital diaphragmatic hernia in rats. J Pediatr Surg 25:426-429, 1990.

Van der Klis FRM, Schmidt EDL, van Beeren HC, Wiersinga WM: Competitive inhibition of T3 binding to α1 and ß1 thyroid hormone receptors by fatty acids. Biochem Biophys Res Comm 179:1011-1016, 1991.

Vidal-Ingigliardi D, Raibaud O: A convenient technique to compare the efficiency of promoters in Escherichia coli. Nucleic Acids Res 13:5919-5926, 1985.

Wiersinga WM: Nuclear thyroid hormone receptors. Neth J Med 28:74-82, 1985.

Chapter 7

CONGENITAL DIAPHRAGMATIC HERNIA: NEW MODELS, NEW IDEAS.

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Ped Surg Int 1994, In press

CONGENITAL DIAPHRAGMATIC HERNIA: NEW MODELS, NEW IDEAS.

7.1 Clinical background

Congenital diaphragmatic hernia (CDH) is an anomaly with unknown etiology and an incidence of 1:3000 liveborns. Despite progress and tremendous effort in neonatal and surgical intensive care the mortality rate has not changed in the last 30 years and remains 30-50%. The outcome of these patients depends mainly on the gravity of pulmonary hypoplasia or on the combination of pulmonary hypoplasia with persistent pulmonary hypertension (Molenaar et al. 1991; Wenstrom et al. 1991).
Structural changes have been demonstrated in the pulmonary parenchyma of patients such as: delayed maturation of alveolar structures (Nakamura et al. 1991) and a decreased number of bronchial branches (Kitagawa et al. 1971). Not only the lung parenchyma but also the pulmonary vascular system shows abnormalities in CDH patients. A decrease in total size of the pulmonary vascular bed, increased thickness of the pulmonary arterial smooth muscle coat, and a decreased number of vessels per unit of lung is found (Geggel et al. 1985; Shochat 1987). In the last 5 years, different clinical approaches have been used in an attempt to influence the overall mortality in CDH patients. These include delayed surgery, alternative ways of artificial ventilation, extracorporeal membrane oxygenation (ECMO), and exogenous surfactant application in selected patients. Even intrauterine repair and postnatal lung transplantation have been advocated in this respect.

7.2 Clinical perspectives and research questions

In neonatology, prematurely born infants with respiratory distress syndrome are treated by modulating lung differentiation by application of corticosteroids starting before birth to enhance the type II alveolar epithelial cells to increase surfactant production. In addition to glucocorticoids, thyroid hormone is another known trophic factor modulating lung growth (Hitchcock 1979). Recently thyrotropin-releasing hormone has been suggested to stimulate pulmonary differentiation (Devaskar et al. 1991; De Zegher et al. 1992). Therefore, fetal hormonal modulation improving lung growth capacity might be preferable to fetal surgical closure of the diaphragmatic defect. However, when the liver, bowel loops, and developing lung parenchyma compete for space in the fetus, it is doubtful whether growth factors should be given to the pregnant mother to stimulate lung growth in prenatally

diagnosed CDH patients. To combine fetal surgery with prenatal enhancement of lung growth by growth factors, either by systemic or local application, is one of the options to be investigated in the near future.
As soon as the newborn with CDH is artificially ventilated, oxygen free radicals will have a major effect on lung parenchyma. Two therapeutic options are available, being primary ECMO treatment (Cornish et al. 1987; O'Rourke et al 1991; Wilson et al. 1991) or application of antioxidant enzymes (Tanswell and Freeman 1987). Primary ECMO treatment of all patients with CDH to prevent oxygen-induced lung damage will however result in overtreatment of at least 50% of CDH patients who would survive without ECMO treatment and escape its intrinsic hazards (intraventricular haemorrhage, carotic ligation). Intratracheal instillation of antioxidant enzymes like polyethyleenglycol bound superoxide dismutase or catalase might be considered as a less aggressive way of treatment with comparable results.
Experimental studies in pigs are now performed to evaluate the possibility of neonatal lung transplantation to overcome the problem of pulmonary hypoplasia. The actual size of the transplanted lung, the best lobe to choose and whether it should be a living related transplantation (parent-child) are currently under study (Shochat, personal communications). In this approach ECMO can be considered as a bridge to transplantation; case reports have documented the use of ECMO for this purpose.

7.3 **Experimental induction of CDH**

In the classical view of treating patients with CDH the abdominal viscera were removed out of the thorax as soon as possible after birth to provide space for the lungs to expand. According to this concept the group of Harrison performed a number of classical studies with pregnant sheep, inducing CDH by means of intrauterine operative techniques in the fetuses (Harrison et al. 1980; 1981). Their aim was to generate an animal model representing CDH appropriate for intrauterine repair enabling enhancement of lung growth. Several disadvantages accompany this sheep model such as surgical interference in a relatively late stage of lung development, high costs for small numbers, while information concerning the pathogenesis of CDH can not be obtained. More recently Glick and coworkers used this animal model to study the functional characteristics of the hypoplastic lung in relation to surfactant production (Glick et al. 1992).
A different approach to study the pathogenesis of CDH with respect to morphology and functional activity of the lung is used by different groups including our own group (Alfonso et al. 1993; Iritani 1984; Kluth et al. 1990; Suen et al. 1993; Ueki et al. 1990). This approach is based on the use of a teratogenic agent known to interfere with lung and diaphragm development.

7.4 **Toxicological background**

2,4-Dichlorophenyl-p-nitrophenyl ether (Nitrofen) has been known as a teratogen with effects on the diaphragm and lung both in rats and mice (Costlow and Manson 1981; Iritani 1984; Kimbrough et al. 1974; Lau et al. 1988; Stone and Manson 1981; Ueki et al. 1990).

Experiments with radioactively labelled Nitrofen have shown that only 1-5% of Nitrofen passes the placenta; Nitrofen radioactivity was detected in the embryonic tissue compartment from 3 hours after application to the mother reaching peak concentrations at 72 hours (Manson 1986). Nitrofen is degraded to hydroxylation, nitroreduction, and acetylation products, which do not show any teratogenic effect by itself (Manson 1986). In the embryo only the native compound can be detected (Manson 1986). In other words the teratogenic effect is not mediated via generation of mutagenic intermediates through nitroreduction of the parent compound.

Experimental data have shown that the effect of Nitrofen is highly dependent on the gestational age of the fetus (Costlow and Manson 1981). The most susceptible period with respect to interference with lung development is from day 9-12 in the rat, which is the actual stage of early lung development. Ueki has described a decreased weight gain of the lung primordium as early as 3 days after experimental application of Nitrofen on day 11 (Ueki et al. 1990); our own group found a significant reduction in growth of the lung primordium also three days after Nitrofen-exposure (Piersma et al. 1993).

The stereochemical configuration of Nitrofen resembles that of triiodothyronine (T_3) and thyroxine (T_4) to a great extent while application of Nitrofen to pregnant mother rats resulted in a decrease in thyrotropin-stimulating hormone and T_4, without major alterations of T_3 in the mothers, as well as a decrease of T_4 in the fetuses (Gray and Kavlock 1983; Manson et al. 1984). Combined application of Nitrofen and T_4 to rats that had been thyroidectomised resulted in 70% reduction of the frequency of congenital anomalies in the offspring (Manson et al. 1984).

In vitro studies performed by our research group have shown that incubation of nuclear T_3 receptor proteins with T_3 in the presence of Nitrofen decreased the specific binding of T_3 to the α_1 and $ß_1$ form of the thyroid hormone receptor (Brandsma et al. 1993b). The effect was dose-dependent (10 μM - 1000 μM). Scatchard analysis revealed that Nitrofen decreased the maximal binding capacity of the receptor while Lineweaver-Burk analysis demonstrated the inhibition to be non-competitive in character. We concluded that Nitrofen inhibits the binding of T_3 to the α_1 and $ß_1$ form of the nuclear T_3-receptor in a non-competitive way by decreasing the maximal binding capacity.

Consequently, lung hypoplasia might result from a decreased binding of T_3 to its receptor.

7.5 Experimental approach

General methods

Adult Sprague-Dawley rats were mated overnight at our laboratory animal center; the next day was designated day 1. On day 10 of pregnancy, a single dose of 100 mg Nitrofen dissolved into olive oil was given to the mother rats through a stomach tube, resulting in large left-sided defects in about 80-90% of the offspring. Previously, we have been using 115 mg/kg body weight at day 11.5 of gestation, which resulted in smaller right-sided defects in about 60% of the offspring. Pregnancy was continued without specific measurements, food and water were supplied ad libitum. Spontaneous delivery was awaited (term=day 22-23), or Caesarean section was performed. After "birth" body weights were determined; at autopsy the presence of a diaphragmatic defect, the position (right- or left-sided), and size of the defect, as well as the nature of the intrathoracic contents (liver, bowel) were evaluated.

Hypoplasia of the lungs

To evaluate the presence of pulmonary hypoplasia after exposure to Nitrofen, newborn rats were weighed and killed by intraperitoneal injection of an overdose of pentobarbital (Nembutal; Sanofi, Tinderberg, The Netherlands). The lungs were dissected out and left and right wet lung weights were determined using a Mettler balance enabling calculation of the lung weight/body weight index. To prevent collapse of the lungs, tracheal cannulation and fixation with Davidson solution (40 vol% ethanol 100%; 5 vol% acetic acid 96%; 10 vol% formaldehyde 37%; 45 vol% saline; pH 7.3) under constant pressure (20 cm H_2O) was performed according to Burri (Burri et al. 1974) for at least 24 hours. The lungs were embedded in paraffin; six micron slides were made and stained with hematoxylin and eosin. Morphometric analysis to determine radial saccular count was performed on at least 3 different slides using a magnification of 250 x. At least 10 radial saccular counts per slide were evaluated. Radial saccular counts were plotted against lung weight/body weight index according to Askenazi (Askenzi and Perlman 1979).

The mean body weights and lung weights (including standard deviations) as well as the lung weight/body weight ratio and the right lung/left lung weight ratio have been published in detail before (Tenbrinck et al. 1990). At term the mean body weight in the Nitrofen-exposed group with CDH (4.8 g $\pm$ 0.3) differed significantly from that in the control group (5.7 g $\pm$ 0.3). The same holds true for total lung weight (Nitrofen-exposed group with CDH: 91.9 mg $\pm$ 24, control group: 151.7 mg $\pm$ 17.3) and for radial saccular counts. In all cases both left and right lungs were significantly smaller in the Nitrofen-exposed group with CDH than in the control group. Plotting of the radial saccular counts against the lung weight/body weight indexes indicated severe hypoplasia of the lung in cases with CDH. Intermediate values were found in the Nitrofen-exposed group without CDH.

Architecture of the lung and alveolar cell composition

To compare the architecture and alveolar epithelial cell composition of the pulmonary acinus in hypoplastic and normal fetal lungs, sections (5 μm) from lungs of control and Nitrofen-exposed fetal rats aged 18-22 days were stained with hematoxylin and eosin. To identify developing alveolar epithelial cells, fresh-frozen sections (6 μm) were incubated with anti-Surfactant Protein A (SP-A; rabbit anti-mouse) or preimmunization serum (indirect immunofluorescence). Criteria to identify immature and mature type II alveolar epithelial cells are: approximately cuboid shape, large and roundish nucleus and cytoplasmic fluorescent staining for SP-A (Ten Have-Opbroek 1981; 1991). Developing type I cells (deriving from type II cells) are low cuboid to squamous, and finally lack SP-A (Ten Have-Opbroek 1981; 1991).

On days 18 and 19, control and Nitrofen-exposed lungs were in the pseudoglandular stage of lung development and looked similar. The prospective pulmonary acinus consisted of tubules with small round lumens, lined by approximately cuboid, fluorescent epithelial cells with large and roundish nuclei (acinar tubules) (Ten Have-Opbroek 1991). On day 20 (canalicular stage), some tubules were slightly dilated and lined by cuboid and thinner fluorescent cells; these tubules were less numerous in exposed lungs. On days 21 and 22, exposed and control lungs were in the saccular stage of lung development. In exposed lungs from fetuses with and without CDH the entire saccular lining consisted of cuboid and low cuboid to thin fluorescent cells, whereas in control lungs fluorescent (low) cuboid cells were interspersed with a few (day 21) or many (day 22) dark zones. In the exposed lungs from fetuses with CDH, the lumens of air spaces were frequently slit-like, septa were thicker, and acinar tubules were more abundant. These phenomena gave the exposed lungs a primitive, compact aspect.

In conclusion, Nitrofen-exposed, and thus hypoplastic, fetal rat lungs are retarded with respect to the differentiation of cuboid type II cells into squamous type I cells whether or not CDH is present and with respect to the development of the future air spaces between days 20 and 22 if CDH is present (Brandsma et al. 1992).

Ultrastructural features of alveolar epithelial cells

To investigate the ultrastructural morphology of type I and type II cells, our light microscopical studies were expanded with electron microscopy of lung tissue from control and Nitrofen-exposed fetal rats aged 19-22 days. Semithin (1 μm) Epon sections were stained with toluidine blue, and ultrathin sections (ca. 80 nm) were stained with uranyl acetate 7% and lead citrate. Electron microscopical features were described and interpreted according to Ten Have-Opbroek (Ten Have-Opbroek et al. 1988; 1990).

On day 19 both control and Nitrofen-exposed lungs contained only cuboid alveolar epithelial cells. From day 20 we found cuboid, low cuboid and thinner epithelial cells. The (low) cuboid cells contained large glycogen fields, some precursory stages of multilamellar bodies (MLBs), and just a few mature MLBs on days 19 and 20; smaller

glycogen fields, more precursory stages and more mature MLBs on day 21; and little or no glycogen but many precursory stages and mature MLBs on day 22. The thinner cells contained little or no glycogen and a few precursory stages of MLBs on days 20 through 22; very thin cells on day 22 contained neither glycogen nor any precursory or mature stages of MLBs. MLBs and tubular myelin were seen in the lumens of future air spaces from day 20 onward.
Nitrofen-exposed lungs differed from control lungs in that inclusion bodies were less abundant in (low) cuboid alveolar cells on days 19 and 20, and more glycogen was seen on day 22. In other words, type II cells were less mature than in control lungs (Brandsma et al. 1993a).

The pulmonary vasculature
Microscopy: For the evaluation of the pulmonary arterial vessels a heated barium gelatine solution was perfused into the pulmonary arterial trunk under constant pressure. The perfusion was stopped when this white solution reached the visceral pleura in all segments leading to 'snow flocks'. Subsequently, tracheal cannulation was performed as described above for fixation of the lung. With the aid of an ocular eye piece micrometer and constant magnification (x250) the pulmonary vessels were evaluated with respect to the external diameter, medial wall thickness and muscularity of the vessel wall.
Both at the level of conducting airways as well as at the level of the terminal bronchioles differences were found consisting of a peripheral extension of the muscular arteries in the hypoplastic lung together with an increase in medial wall thickness (Tenbrinck et al. 1992).

Eicosanoids: The eicosanoid content in bronchoalveolar lavage (BAL) fluid from Nitrofen-exposed neonatal rats with CDH and control rats was determined. In one group, BAL was performed after Casarean section on day 22 before any breathing movement could take place. In another group, BAL was performed directly after spontaneous birth. Contents of 6-keto-prostaglandin-$F_{1\alpha}$ (6-k-$PGF_{1\alpha}$) and thromboxane B_2 (TxB_2) were measured in BAL by radioimmuno assay.
In control rats, the mean concentration of 6-k-$PGF_{1\alpha}$ (with a known vasodilatory effect) in BAL was significantly higher after spontaneous birth than after Caesarean section, whereas in rats with CDH this increase could not be demonstrated. The mean concentration of TxB_2 (a vasoconstrictive agent) increased significantly during birth in the Nitrofen-exposed group with CDH, but not in de control group; despite the increase during birth, TxB_2 concentrations in the CDH group were significantly lower than those in the control group. These phenomena might play a role in the occurrence of pulmonary hypertension in CDH, although we realize that the eicosanoid content of BAL does not necessarily reflect that of the pulmonary vasculature (IJsselstijn et al. 1994).

Pulmonary neuroendocrine cells (PNEC): Amine and peptide producing PNEC, widely distributed throughout airway mucosa, are thought to play an important role in both pulmonary development and regulation of pulmonary vascular tone. Furthermore, recent studies show modulation during chronic hypoxia of calcitonin gene-related peptide (CGRP), a pulmonary vasodilator produced by PNEC (Springall and Polak 1993). Morphometric analysis of CGRP immunoreactive PNEC clusters, i.e. neuroepithelial bodies (NEB), was performed in sections of lungs from Nitrofen-exposed neonatal rats with CDH and control rats. NEB size, and number of NEBs/area of lung were assessed using a semiautomatic image analysis system.
In lungs from Nitrofen-exposed rats with CDH, there is a significant increase in relative number of NEBs per surface area of lung parenchyma compared to lungs from control rats, whereas the size of NEBs is not significantly affected. Whether this results in altered NEB function including imbalance in vasoactive mediators requires further studies.

Functional studies
To evaluate the functional significance of the abnormal differentiation of the alveolar epithelial cells the following studies were performed.

Surfactant production: In bronchoalveolar lavage fluid of control and Nitrofen-exposed fetuses (day 22) phospholipid fractions and SP-A content were measured. Significantly lower total amounts of phospholipids were recovered from Nitrofen-exposed rats by bronchoalveolar lavage than from control rats. However, correction for lung weight and bronchoalveolar lavage efficiency may reduce the diverging results. The phospholipid pool had the same composition, and SP-A per mol phospholipid was the same in bronchoalveolar lavage fluid from Nitrofen-exposed rats as in that from control rats (Brandsma et al. 1993a).

Surfactant and artificial ventilation: Preliminary results in newborn infants with CDH suggested a positive effect of surfactant administration on oxygenation (Bos et al. 1991). To investigate the effect of surfactant on ventilation parameters in our rat model, newborn rats were intubated immediately after birth, transferred to a heated multi-chambered body plethysmograph, and artificially ventilated. Peak inspiratory pressures (PIP) were initially set at 17 cm H_2O without any positive end expiratory pressure (PEEP), and a fraction of inspired oxygen (FiO_2) of 1.0. The pressure was raised in steps of 5 cm H_2O from 5 to 30 cm H_2O to obtain pressure/volume diagrams after 0, 1, and 6 hours of artificial ventilation (Lachmann et al 1981). These measurements were obtained with and without endotracheal instillation of 0.05 ml bovine surfactant (25 mg/ml) in control rats and in Nitrofen-exposed rats with CDH.
Significant differences in tidal volume were observed between control rats and Nitrofen-exposed rats at all time intervals. A positive effect of surfactant application on tidal

volume was found in control rats at t=1 hour. No significant differences were observed between the Nitrofen-exposed groups with or without surfactant at t=1 or t=6 hours (Scheffers et al. 1994). Distribution studies with labelled surfactant will be necessary to evaluate the spread of surfactant in both lungs.

Reaction of the lung on artificial ventilation: Histological examination of neonatal rat lungs revealed that after ventilation with PIP of 17 cm H_2O no aeration of lungs was present in control rats and Nitrofen-exposed rats with CDH. The lungs could be aerated with PIP of 25 cm H_2O. To evaluate optimal ventilatory strategy, the clinical and histological aspects were studied using two different ventilatory settings. Pressure-controlled artificial ventilation started immediately after birth and was continued for six hours. Group 1 was ventilated with PIP of 25 cm H_2O all the time; group 2 with PIP of 25 cm H_2O for 15 minutes followed by PIP of 17 cm H_2O for the rest of the time. Other settings for both groups were: PEEP 3 cm H_2O, freq. 40/min, and FiO_2 1.
In group 1, 10 out of 11 rats with CDH and 6 out of 10 control rats died due to pneumothorax. In group 2, 20 out of 53 rats with CDH died of pneumothorax, whereas all 33 control rats survived. Histological investigation revealed a centroacinar pattern of lung expansion in lungs from rats with CDH. Aeration was normal in nearly 60% of the control rats after 6 hours of artificial ventilation.
We conclude that in lungs from control rats and from rats with CDH high opening pressures are needed, but continuous PIP of 25 cm H_2O results in high mortality due to pneumothorax, especially in rats with CDH. The centroacinar lung expansion and the incidence of pneumothorax in CDH rats is similar to the situation seen in premature children with respiratory distress syndrome and primary or secondary surfactant deficiency.

Antioxidant enzyme activity: In another series of experiments the lungs from fetal rats (days 19, 20, 21, and 22) were examined for protein and DNA content and activity/mg DNA of superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX). The same parameters were assessed in tracheotomized newborn rats after pressure-controlled artificial ventilation with either room air or 100% oxygen during a short period of 5 hours.
Both in Nitrofen-exposed rats with CDH and in control rats, wet lung weight increased during gestation. At term, rats with CDH had significantly lower mean lung weights than control rats. Neither group differed in protein and DNA content per mg lung or SOD, catalase, and GPX activity per mg DNA before and at birth. After artificial ventilation of neonates with room air and 100% oxygen, SOD activity tended to decrease, whereas catalase activity remained virtually unchanged in the lungs from rats with CDH. However, GPX activity in these lungs was reduced to 80% of initial activity at term after ventilation with air and to 70% after ventilation with 100% oxygen. The present finding of a decline in GPX activity in this animal model after a short period of artificial ventilation may

indicate that the rat neonate with CDH is at risk to develop oxygen related lung damage (Sluiter et al. 1992).

7.6 **Research Perspectives**

The remaining research questions which should be answered using the different animal models to elucidate the etiology and pathogenesis of CDH are summarized in the table:

Aspects of pulmonary development

Morphogenesis
- interaction lung bud - mesenchyme
- branching pattern
- vasculature - bloodflow
- relationship with heart, liver, diaphragm
- closing of the pleuroperitoneal canals

Differentiation
- epithelial changes
  * type I and type II cells
- hormonal influences
  * thyroxine
  * glucocorticoids
- antioxidant enzymes
- release of vasoactive substances

With respect to the lung, the understanding of morphogenesis relies on knowledge of cell movement, cell adhesion, cell growth, and cellular differentiation. Epithelial-mesenchymal interactions play key roles in these processes, while extracellular matrix molecules regulate mesenchymal-epithelial interaction during organ formation. A number of growth factors such as epidermal growth factor, fibroblast growth factor and others are known to be involved in cellular proliferation and differentiation (Warburton et al. 1993).
Use of serumless chemically defined organ culture systems, transgenic mice, or other models can possibly contribute to further basic understanding of pulmonary development. Pediatric surgeons have the unique opportunity to share their knowledge of congenital anomalies such as congenital diaphragmatic hernia with knowledge and skills gained by scientists in laboratories of cell biology and genetics.

7.7 References

Alfonso LF, Vilanova J, Aldazabal P, Lopez de Torre B, Tovar JA: Lung growth and maturation in the rat model of experimentally induced congenital diaphragmatic hernia. Eur J Pediatr Surg 3:6-11, 1993.

Askenazi SS, Perlman M: Pulmonary hypoplasia: lung weight and radial alveolar count as criteria of diagnosis. Arch Dis Child 54:614-618, 1979.

Bos AP, Tibboel D, Hazebroek FWJ, Molenaar JC, Lachman B, Gommers D: Surfactant replacement therapy in high-risk congenital diaphragmatic hernia. (Letter to editor) Lancet 338:1279, 1991.

Brandsma AE, Ten Have-Opbroek AAW, Vulto IM, Molenaar JC, Tibboel D: Alveolar epithelial composition and architecture in a rat model of lung hypoplasia. Am Rev Resp Dis 145(Suppl):A126, 1992.

Brandsma AE, Tibboel D, Vulto IM, Egberts J, Ten Have-Opbroek AAW: Ultrastructural features of alveolar epithelial cells in the late fetal pulmonary acinus: A comparison between normal and hypoplastic lungs using a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia. Microsc Res Techn 26:389-399, 1993a.

Brandsma AE, Tibboel D, Vulto IM, Ten Have-Opbroek AAW, Wiersinga WM: Lung hypoplasia is induced by Nitrofen: an effect via inhibition of T_3-receptor binding? Am Rev Resp Dis 147(Suppl):A415, 1993b.

Burri PH, Dbaly J, Weibel ER: The postnatal growth of the rat lung. I. Morphometry. Anat Rec 178:711-730, 1974.

Cornish JD, Gerstmann DR, Clark RH, Carter JM, Null DM Jr, deLemos RA: Extracorporeal membrane oxygenation and high frequency oscillatory ventilation: potential therapeutic relationships. Crit Care Med 15:831-834, 1987.

Costlow RD, Manson JM: The heart and diaphragm: Target organs in the neonatal death induced by Nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether). Toxicology 20:209-227, 1981.

Devaskar UP, deMello DE, Ackerman J: Effect of maternal administration of thyrotropin-releasing hormone or DN1417 on functional and morphologic fetal rabbit lung maturation and duration of survival after premature delivery. Biol Neonate 59:346-351, 1991.

De Zegher F, Spitz B, De Vlieger H: Prenatal treatment with thyrotrophin releasing hormone to prevent neonatal respiratory distress. Arch Dis Child 67:231-237, 1992.

Geggel RL, Murphy JD, Langleben D, Crone RK, Vacanti JP, Reid LM: Congenital diaphragmatic hernia: Arterial structural changes and persistent pulmonary hypertension after surgical repair. J Pediatr 107:457-464, 1985.

Glick PL, Stannard VA, Leach CL, Rossman J, Hosada Y, Morin FC, Cooney DR, Allen JE, Holm B: Pathophysiology of congenital diaphragmatic hernia II: The fetal lamb CDH model is surfactant deficient. J Pediatr Surg 27:382-388, 1992.

Gray LE, Kavlock RJ: The effects of the herbicide 2,4-dichlorophenyl-p-nitrophenyl ether (Nit) on serum thyroid hormones in adult female mice. Toxicology Letters 15:231-235, 1983.

Harrison MR, Jester JA, Ross NA: Correction of congenital diaphragmatic hernia in utero. I. The model: intrathoracic balloon produces fetal pulmonary hypoplasia. Surgery 88:174-182, 1980.

Harrison MR, Ross NA, deLorimier AA: Correction of congenital diaphragmatic hernia in utero. III. Development of a successful surgical technique using abdominoplasty to avoid compromise of umbilical blood flow. J Pediatr Surg 16:934-942, 1981.

Hitchcock KR: Hormones and the lung: I. Thyroid hormones and glucocorticoids in lung development. Anat Rec 194:15-40, 1979.

IJsselstijn H, Zijlstra FJ, Bos AP, Molenaar JC, Tibboel D: Eicosanoid content in bronchoalveolar lavage fluid in newborn rats with pulmonary hypoplasia and congenital diaphragmatic hernia. Am J Resp Crit Care Med (Suppl):A745, 1994.

Iritani I: Experimental study on embryogenesis of congenital diaphragmatic hernia. Anat Embryol 169:133-139, 1984.

Kimbrough RD, Gaines TB, Linder RE: 2,4-Dichlorophenyl-p-nitrophenyl ether (TOK): Effects on the lung maturation of rat fetus. Arch Environ Health 28:316-320, 1974.

Kitagawa M, Hislop A, Boyden EA, Reid L: Lung hypoplasia in congenital diaphragmatic hernia: a quantative study of airway, artery, and alveolar development. Br J Surg 58:342-346, 1971.

Kluth D, Kangah R, Reich P, Tenbrinck R, Tibboel D, Lambrecht W: Nitrofen-induced diaphragmatic hernias in rats: an animal model. J Pediatr Surg 25:850-854, 1990.

Lachmann B, Grossmann G, Freyse J, Robertson B: Lung-thorax compliance in the artificially ventilated premature rabbit neonate in relation to variations in inspiration:expiration ratio. Pediatr Res 15:833-838, 1981.

Lau C, Cameron AM, Irsula O, Antolick LL, Langston C, Kavlock RJ: Teratogenic effects of Nitrofen on cellular and functional maturation of the rat lung. Toxicol Appl Pharmacol 95:412-422, 1988.

Manson JM: Mechanism of Nitrofen teratogenesis. Environ Health Perspect 70:137-147, 1986.

Manson JM, Brown T, Baldwin DM: Teratogenicity of Nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) and its effects on thyroid function in the rat. Toxicol Appl Pharmacol 73:323-335, 1984.

Molenaar JC, Bos AP, Hazebroek FWJ, Tibboel D: Congenital diaphragmatic hernia, what defect? J Pediatr Surg 26:248-254, 1991.

Nakamura Y, Yamamoto I, Fukuda S, Hashimoto T: Pulmonary acinar development in diaphragmatic hernia. Arch Pathol Lab Med 115:372-376, 1991.

O'Rourke PP, Lillehei CW, Crone RK, Vacanti JP: The effect of extracorporeal membrane oxygenation on the survival of neonates with high-risk congenital diaphragmatic hernia: 45 cases from a single institution. J Pediatr Surg 26:147-152, 1991.

Piersma AH, Hofstee-Hooftman MWA, Verhoef A: Early lung development in the rat and its perturbation by Nitrofen treatment. Teratology 48:31A, 1993.

Scheffers EC, IJsselstijn H, Tenbrinck R, Lachmann B, De Jongste JC, Molenaar JC, Tibboel D: Evaluation of lung function changes before and after surfactant application during artificial ventilation in newborn rats with congenital diaphragmatic hernia (CDH). J Pediatr Surg 29:820-824, 1994.

Shochat SJ: Pulmonary vascular pathology in congenital diaphragmatic hernia. Pediatr Surg Int 2:331-335, 1987.

Sluiter W, Bos AP, Silveri F, Tenbrinck R, Kraakslee R, Tibboel D, Koster JF, Molenaar JC: Nitrofen-induced diaphragmatic hernias in rats: pulmonary antioxidant enzyme activities. Pediatr Res 32:394-398, 1992.

Springall DR, Polak JM: Calcitonin gene-related peptide and pulmonary hypertension in experimental hypoxia. Anat Rec 236:96-104, 1993.

Stone LC, Manson JM: Effects of the herbicide 2,4-dichlorophenyl-p-nitrophenyl ether (Nitrofen) on fetal lung development in rats. Toxicology 20:195-207, 1981.

Suen HC, Catlin EA, Ryan DP, Wain JC, Donahoe PK: Biochemical immaturity of lungs in congenital diaphragmatic hernia. J Pediatr Surg 28:471-477, 1993.

Tanswell AK, Freeman BA: Liposome-entrapped antioxydant enzymes prevent lethal O_2 toxicity in the newborn rat. J Appl Physiol 63(1):347-352, 1987.

Tenbrinck R, Tibboel D, Gaillard JLJ, Kluth D, Bos AP, Lachmann B, Molenaar JC: Experimentally induced congenital diaphragmatic hernia in rats. J Pediatr Surg 25:426-429, 1990.

Tenbrinck R, Gaillard JLJ, Tibboel D, Kluth D, Lachmann B, Molenaar JC: Pulmonary vascular abnormalities in experimentally induced CDH in rats. J Pediatr Surg 27:862-865, 1992.

Ten Have-Opbroek AAW: The development of the lung in mammals: an analysis of concepts and findings. Am J Anat 162:201-219, 1981.

Ten Have-Opbroek AAW: Lung development in the mouse embryo. Exp Lung Res 17:111-130, 1991.

Ten Have-Opbroek AAW, Dubbeldam JA, Otto-Verberne CJM: Ultrastructural features of type II alveolar epithelial cells in early embryonic mouse lung. Anat Rec 221:846-853, 1988.

Ten Have-Opbroek AAW, Otto-Verberne CJM, Dubbeldam JA: Ultrastructural characteristics of inclusion bodies of type II cells in late embryonic mouse lung. Anat Embryol 181:317-323, 1990.

Ueki R, Nakao Y, Nishida T, Nakao Y, Wakabayashi T: Lung hypoplasia in developing mice and rats induced by maternal exposure to Nitrofen. Cong Anom 30:133-143, 1990.

Warburton D, Lee M, Berberich MA, Bernfield M: Molecular embryology and the study of lung development. Am J Respir Cell Mol Biol 9:5-9, 1993.

Wenstrom KD, Weiner CP, Hanson JW: A five-year statewide experience with congenital diaphragmatic hernia. Am J Obstet Gynecol 165:838-842, 1991.

Wilson JM, Lund DP, Lillehei GW et al: Congenital diaphragmatic hernia: predictors of severity in the ECMO era. J Pediatr Surg 26:1028-1034, 1991.

Chapter 8

THE NITROFEN MODEL VERSUS THE HUMAN SITUATION

THE NITROFEN MODEL VERSUS THE HUMAN SITUATION

8.1 The Nitrofen model versus the human situation

As mentioned in the introduction of this thesis, the mortality rate of congenital diaphragmatic hernia (CDH) has not improved in the last 30 years, despite the development of antenatal diagnostic procedures, advanced surgical techniques, and intensive perioperative care. The abnormalities of the lung, i.e. pulmonary hypoplasia and persistent pulmonary hypertension, are the main causes of the high morbidity and mortality rates. To obtain better insight into the pathogenesis of CDH and the resulting histologic abnormalities, we have directed our attention to the early and late fetal lung development and the closing process of the diaphragm in our rat model for CDH and pulmonary hypoplasia. This rat model is based on the use of 2,4-dichlorophenyl-p-nitrophenylether (Nitrofen), a herbicide with teratogenic effects on the development of lung and diaphragm. In addition, we have tried to further elucidate the mode of action of Nitrofen.

In the rat the lung primordium is visible from day 12 of gestation, initially as an outgrowth of the bottom of the foregut, and on day 13 as a triplicate tube growing in caudal direction. After Nitrofen-exposure on gestational day 10, the early growth of the lung, as measured in serial longitudinal sections on day 13, is reduced (Brandsma et al. 1994a). Staining of normal lungs and Nitrofen-exposed lungs of approximately this gestational age with antibodies against the proliferation marker PCNA (proliferating cell nuclear antigen) and against some markers which are known to be of importance for the differentiation of tissues (collagen III and IV, fibronectin, and laminin) does not reveal any differences between the two groups. In other words, using these techniques, no difference in proliferation rate or differentiation signals is demonstrable between normal fetal rat lungs and (smaller!) Nitrofen-exposed fetal rat lungs (this thesis, chapter 5).

Two days later (day 15), clear evidence of disturbed diaphragmatic development leaving parts of the liver uncovered is visible by scanning electron microscopy in Nitrofen-exposed rat embryos (Kluth et al. 1993). On the next days the liver is seen inside the thoracic cavity, and from day 22 displaced bowel loops are present in the thoracic cavity as well. In those rat fetuses with massive liver protrusion, the lungs are hypoplastic, indicating that growth impairment of the lung results from a competition for space in the embryonic thoracic cavity (Kluth et al. 1993).

From at least day 19 onwards, Nitrofen-exposed fetal rat lungs have smaller lung volumes and lung tissue volumes as determined by morphometric techniques (Brandsma et al.

1994b). Between days 20 and 22 these lungs are atelectatic, and retarded with respect to the development of the future air spaces if CDH is present. The differentiation of immature cuboid type II cells into mature type II cells (Brandsma et al. 1993) and into squamous type I cells (Brandsma et al. 1994b) is also retarded, resulting in relatively more type II cells lining the alveolar surfaces. This retardation of type II cell development does not have an obvious impact on the composition of extracellular surfactant (Brandsma et al. 1993).

In neonatal lungs from Nitrofen-exposed rats, a peripheral extension of the muscular arteries together with an increase in medial wall thickness is present both at the level of conducting airways as well as at the level of the terminal bronchioles (Tenbrinck et al. 1992).

As in the Nitrofen model CDH in humans is accompanied by ipsilateral and (to a lesser extent) contralateral hypoplasia of the lungs. Histologic studies describing the different stages during abnormal lung development and the resulting morphology in patients with CDH have diverging results. A lower number of bronchial branches and alveoli, retardation of alveolar development, the presence of more cuboidal cells and atelectasis (Areechon and Reid 1963; Boyden 1972; Campanale and Rowland 1955; George et al. 1987; Kitagawa et al. 1971; Nakamura et al. 1991), and increased muscularity of the pulmonary vascular bed (Beals et al. 1992; Bohn et al. 1987; Geggel et al. 1985; Levin 1978) are demonstrated.

As stated before, the mode of action by which Nitrofen exerts its teratogenic effects is largely unknown. Timing and dosage of the administration of the compound is crucial: Nitrofen administered to rats on day 10 of pregnancy results in leftsided hernias, while administration on day 12 results in rightsided hernias (Kluth et al. 1990); exposure on other days induces heart, kidney, or other malformations (Burke Hurt et al. 1983; Costlow and Manson 1981; Gray et al. 1983; Lau et al. 1986; Ostby et al. 1985). Apparently, the teratogenic time window differs per organ, and sometimes even within one organ. Results from several research groups (Gray and Kavlock 1983; Manson et al. 1984; Manson 1986) are suggesting an interference of Nitrofen with thyroid hormone function. The stereochemical configuration of Nitrofen resembles that of triiodothyronine (T_3) and thyroxine (T_4) to a great extent while application of Nitrofen to pregnant mother rats resulted in a decrease in thyrotropin-stimulating hormone and T_4, without major alterations of T_3 in the mothers as well as a decrease of T_4 in the fetuses (Gray and Kavlock 1983; Manson et al. 1984). Combined application of Nitrofen and T_4 to rats that had been thyroidectomised resulted in 70% reduction of the frequency of congenital anomalies in the offspring (Manson et al. 1984). Our own in vitro experiments show that Nitrofen inhibits the binding of triiodothyronine (T_3) to the nuclear T_3-receptor (Brandsma et al. 1994c). Consequently, Nitrofen-inducable defects, such as CDH and pulmonary hypoplasia, might result from a decreased binding of T_3 to its receptor. A possible explanation for a teratogenic time window is that the expression of the nuclear T_3-receptor

differs in time per organ. These studies are currently under way in our laboratory using immunohistochemistry and in situ hybridization.

It is known that thyroid hormone plays a significant role during the last phases of lung development, especially on the surfactant producing type II alveolar epithelial cells. This has long been assumed the only effect of thyroid hormone on pulmonary development. However, based on our own results (Brandsma et al. 1994c), and on the fact that transfer of thyroid hormone through the placenta takes place in humans (Vulsma et al. 1989), a role of thyroid hormone during early fetal lung development can not be excluded.

Knowledge of the mode of action of Nitrofen may eventually unravel the course of events in humans, and provide directions for the therapy or even the prevention of this serious anomaly.

In a study on the etiological aspects of CDH in humans, Bos et al. (1994) do not show any association with a list of teratogens, nor with maternal thyroid dysfunction. They approached all parents of children with CDH admitted to their pediatric surgical intensive care unit from 1984 to 1990. Questions were asked related to potential teratologic data. In addition, blood was drawn immediately postpartum from nine mothers, and maternal antibodies were determined against thyroglobulin, thyroid microsomes, and thyroid stimulating hormone receptors. No specific association was found to be significant. A large population-based, case-control study of Khoury et al. (1989) showed no relationship between the risk of birth defects and history of maternal hypothyroidism either.

In conclusion, as long as the etiology of CDH and pulmonary hypoplasia is unknown, we have to be very careful with the extrapolation of knowledge obtained in the rat model to the human situation, even when the model seems as perfect as the Nitrofen model does.

8.2 References

Areechon W, Reid L: Hypoplasia of the lung with congenital diaphragmatic hernia. Br Med J 1:230-233, 1963.

Beals DA, Schloo BL, Vacanti JP, Reid LM, Wilson JM: Pulmonary growth and remodeling in infants with high-risk congenital diaphragmatic hernia. J Pediatr Surg 27:997-1002, 1992.

Bohn D, Tamura M, Perrin D, Barker G, Rabinovitch M: Ventilatory predictors of pulmonary hypoplasia in congenital diaphragmatic hernia, confirmed by morphologic assessment. J Pediatr 111:423-431, 1987.

Boyden EA: The structure of compressed lungs in congenital diaphragmatic hernia. Am J Anat 134:497-507, 1972.

Bos AP, Pattenier AM, Grobbee RE, Lindhout D, Drexhage HA, Tibboel D, Molenaar JC: Etiological aspects of congenital diaphragmatic hernia: results of a case comparison study. Hum Genet 94:445-446, 1994.

Brandsma AE, Tibboel D, Vulto IM, Egberts J, ten, Have-Opbroek AAW: Ultrastructural features of alveolar epithelial cells in the late fetal pulmonary acinus. A comparison between normal and hypoplastic lungs, using a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia. In: Microscopic evaluation of respiratory tract function. Ten Have-Opbroek AAW and Plopper CG, Eds. Microsc Res Techn 26:389-399, 1993.

Brandsma AE, Piersma AH, Hofstee-Hooftman MWA, Verhoef A, Ten Have-Opbroek AAW, Tibboel D: Early lung development in the rat and its perturbation by Nitrofen exposure. Pediatr Res 36:47A, 1994a.

Brandsma AE, Ten Have-Opbroek AAW, Vulto IM, Molenaar, J.C., Tibboel D: Alveolar epithelial composition and architecture of the late fetal pulmonary acinus: an immunocytochemical and morphometric study in a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia. Exp Lung Res 20:491-515, 1994b.

Brandsma AE, Tibboel D, Vulto I, de Vijlder JJM, ten, Have-Opbroek AAW, Wiersinga WM: Inhibition of T3-receptor binding by Nitrofen. Biochim Biophys Acta 1201:266-270, 1994c.

Burke Hurt SS, Smith JM, Hayes AW: Nitrofen: a review and perspective. Toxicology 29:1-37, 1983.

Campanale RP, Rowland RH: Hypoplasia of the lung associated with congenital diaphragmatic hernia. Ann Surg 142:176-189, 1955.

Costlow RD, Manson JM: The heart and diaphragm: Target organs in the neonatal death induced by Nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether). Toxicology 20:209-227, 1981.

Geggel RL, Murphy JD, Langleben D, Crone RK, Vacanti JP, Reid LM: Congenital diaphragmatic hernia: Arterial structural changes and persistent pulmonary hypertension after surgical repair. J Pediatr 107:457-464, 1985.

George DK, Cooney TP, Chiu BK, Thurlbeck WM: Hypoplasia and immaturity of the terminal lung unit (acinus) in congenital diaphragmatic hernia. Am Rev Respir Dis 136:947-950, 1987.

Gray LE, Kavlock RJ: The effects of the herbicide 2,4-dichlorophenyl-p-nitrophenyl ether (Nit) on serum thyroid hormones in adult female mice. Toxicol Lett 15:231-235, 1983.

Khoury MJ, Becerra JE, d'Almada PJ: Maternal thyroid disease and risk of birth defects in offspring: a population-based case-control study. Paediatr Perinat Epidemiol 3:401-420, 1989.

Kitagawa M, Hislop A, Boyden EA, Reid L: Lung hypoplasia in congenital diaphragmatic hernia. A quantitative study of airway, artery, and alveolar development. Br J Surg 58:342-346, 1971.

Kluth D, Kangah R, Reich P, Tenbrinck R, Tibboel D, Lambrecht W: Nitrofen-induced diaphragmatic hernias in rats: an animal model. J Pediatr Surg 25:850-854, 1990.

Kluth D, Tenbrinck R, von Ekesparre M, Kangah R, Reich P, Brandsma A, Tibboel D, Lambrecht W: The natural history of congenital diaphragmatic hernia and pulmonary hypoplasia in the embryo. J Pediatr Surg 28:456-463, 1993.

Lau C, Cameron AM, Irsula O, Robinson KS: Effects of prenatal Nitrofen exposure on cardiac structure and function in the rat. Toxicol Appl Pharmacol 86:22-32, 1986.

Levin DL: Morphologic analysis of the pulmonary vascular bed in congenital left-sided diaphragmatic hernia. J Pediatr 92:805-809, 1978.

Manson JM: Mechanism of Nitrofen teratogenesis. Environ Health Persp 70:137-147, 1986.

Manson JM, Brown T, Baldwin DM: Teratogenicity of Nitrofen (2,4,-dichloro-4'-nitrodiphenyl ether) and its effects on thyroid function in the rat. Toxicol Appl Pharmacol 73:323-335, 1984.

Nakamura Y, Yamamoto I, Fukuda S, Hashimoto T: Pulmonary acinar development in diaphragmatic hernia. Arch Pathol Lab Med 115:372-376, 1991.

Ostby JS, Gray LE, Kavlock RJ, Ferrell JM: The postnatal effects of prenatal exposure to low doses of Nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) in Sprague-Dawley rats. Toxicology 34:285-297, 1985.

Tenbrinck R, Gaillard JLJ, Tibboel D, Kluth D, Lachmann B, Molenaar JC: Pulmonary vascular abnormalities in experimentally induced CDH in rats. J Pediatr Surg 27:862-865, 1992.

Vulsma T, Gons MH, de Vijlder JJM: Maternal-fetal transfer of thyroxine in congenital hypothyroidism due to a total organification defect or thyroid agenesis. N Engl J Med 321:13-16, 1989.

SUMMARY

SUMMARY

This study was undertaken to investigate the pathogenetic aspects of pulmonary hypoplasia in congenital diaphragmatic hernia (CDH). CDH is a birth defect in which the organs of the abdominal cavity herniate into the chest cavity through an incompletely closed diaphragm. It is accompanied by unilateral or bilateral pulmonary hypoplasia with resultant respiratory failure after birth in the majority of the cases. The incidence of CDH is 1:3000 liveborns, the etiology is unknown, and the mortality rate is 30-50%.
For this study we used a rat model of CDH and pulmonary hypoplasia induced by the herbicide 2,4-dichlorophenyl-p-nitrophenyl (Nitrofen).

Chapter 1 introduces the problem of CDH and pulmonary hypoplasia. An outline of literature data on normal and abnormal development of fetal human and rat lungs is given.
In **chapter 2** the alveolar epithelial composition and architecture of the late fetal pulmonary acinus in hypoplastic and normal rat lungs is described. In this study we used lung tissue from normal control and Nitrofen-exposed fetal Sprague Dawley rats aged 18-22 days. Sections were stained with hematoxylin and eosin or incubated with anti-Surfactant Protein A (SP-A) antiserum to identify developing alveolar epithelial cells, and studied by light microscopy. Morphometric techniques were used to quantitate the differences between the control and the Nitrofen-exposed groups. We found that Nitrofen-exposed fetal rat lungs (1) have smaller lung volumes and lung tissue volumes from at least day 19 onwards; (2) are retarded with respect to the differentiation of cuboid type II cells into squamous type I cells; (3) have smaller total volumes of type II cells if CDH is present, but their alveolar surfaces are lined by relatively more type II cells and less type I cells whether or not CDH is present; (4) are atelectatic, and retarded with respect to the development of the future air spaces between days 20 and 22 if CDH is present.
To study the ultrastructural features and functional maturity of the alveolar epithelial cells in hypoplastic and normal fetal rat lungs, we used the transmission electron microscope, and determined phospholipid fractions and SP-A content in bronchoalveolar lavage fluid. The results are described in **chapter 3**. We found that there are less mature multilamellar bodies (MLBs) on day 20 and more glycogen on day 22 in type II cells of Nitrofen-exposed lungs, suggesting a retarded maturation of these type II cells in hypoplastic lungs compared to those in control lungs. The phospholipid composition as well as the amount of SP-A per mol phospholipid in bronchoalveolar lavage fluid from fetal rat lungs is the same in both the Nitrofen-exposed and the control group. Thus, the composition of extracellular surfactant is probably the same in both groups.

In **chapter 4** the natural history of CDH associated with pulmonary hypoplasia is studied by scanning electron microscopy. We found that impaired lung development is proportional to the size of the liver mass inside the thoracic cavity. This indicates that growth impairment is not the result of lung compression in the fetus but rather the result of growth competition in the embryo: the liver that grows faster than the lung reduces the available space. If the remaining space is too small, pulmonary hypoplasia will result.

In the next study, described in **chapter 5**, we have tried to distinguish between effects of Nitrofen on early lung proliferation and on early lung differentiation. As criteria for proliferation were taken: measurements of length of the pulmonary primordium and staining for proliferating cell nuclear antigen (PCNA). As differentiation markers we used antibodies against the collagens III and IV, laminin, and fibronectin. It was shown that the early growth of the lung primordium was reduced on day 13 after exposure to Nitrofen on day 10. No differences between normal and Nitrofen-exposed fetal rat lungs were found using the other markers.

Data from the literature suggest that Nitrofen might exert its teratogenic action via an alteration of thyroid hormone metabolism. Therefore, we investigated the effect of Nitrofen on the binding of thyroid hormone to the α_1 and β_1 form of the thyroid hormone receptor obtained from bacteria by recombinant DNA techniques. We found that Nitrofen decreases the binding of T_3 to the thyroid hormone receptor in a non-competitive way. Consequently, rat lung hypoplasia might result from the decreased binding of T_3 to its receptor after exposure to Nitrofen. This study is described in **chapter 6**.

Chapter 7 offers a review and discussion of the most important results so far obtained in our Nitrofen rat model, and formulates some clinical perspectives and research questions.

Finally, in **chapter 8**, a comparison is made between the human situation and the Nitrofen model for CDH and pulmonary hypoplasia. It is concluded that we have to be careful with the extrapolation of knowledge obtained in the rat model to the human situation, even when the model seems as perfect as the Nitrofen model does.

SAMENVATTING

SAMENVATTING

In dit proefschrift wordt verslag gedaan van een onderzoek naar de pathogenetische aspecten van longhypoplasie bij congenitale hernia diaphragmatica (CDH). CDH is een aangeboren afwijking waarbij de buikorganen via een defect in het middenrif in de borstholte herniëren. In vrijwel alle gevallen gaat dit gepaard met uni- of bilaterale longhypoplasie en ernstige ademhalingsproblemen na de geboorte. CDH komt voor bij 1 op de 3000 pasgeborenen, de etiologie is onbekend en de mortaliteit is 30 tot 50%.
Voor deze studie hebben we gebruik gemaakt van een diermodel: toediening van het landbouwgif 2,4-dichlorophenyl-p-nitrophenyl (Nitrofen) aan zwangere ratten resulteert in CDH en longhypoplasie bij de nakomelingen.

In **hoofdstuk 1** wordt het probleem van CDH en longhypoplasie geïntroduceerd. Het bevat een overzicht van gegevens uit de literatuur over normale en abnormale ontwikkeling van de foetale long bij de mens en bij de rat.
In **hoofdstuk 2** wordt de samenstelling van het alveolaire epitheel en de architectuur van de laat-foetale longacinus in hypoplastische en normale rattelongen beschreven. Voor deze studie hebben we longweefsel van normale en van aan Nitrofen blootgestelde foetale Sprague Dawley ratten gebruikt, varierend in leeftijd van 18 tot 22 dagen. Van dit longweefsel werden coupes gesneden en gekleurd met hematoxyline en eosine of geïncubeerd met anti Surfactant Protein A (SP-A) antiserum om alveolaire epitheliale cellen te herkennen. Morfometrische technieken werden gebruikt om de verschillen tussen de controlegroep en de Nitrofen-groep te kwantificeren. We hebben gevonden dat aan Nitrofen blootgestelde foetale rattelongen (1) kleinere longvolumina en longweefsel-volumina hebben tenminste vanaf dag 19; (2) een vertraagde differentiatie van kubische type II cellen in platte type I cellen hebben; (3) een kleiner totaal volume van type II cellen hebben als er een hernia aanwezig is, maar dat het alveolaire oppervlak bekleed is met relatief meer type II cellen en minder type I cellen ongeacht de aanwezigheid van een hernia; (4) atelectatisch zijn, en vertraagd wat betreft de ontwikkeling van de toekomstige luchtruimten tussen dag 20 en 22 als er een hernia aanwezig is.
Om de ultrastructuur en de functionele rijpheid van de alveolaire epitheliale cellen in hypoplastische en normale foetale rattelongen te bestuderen hebben we gebruik gemaakt van de transmissie electronenmicroscoop. Tevens hebben we de fosfolipide fracties en het SP-A gehalte in bronchoalveolaire lavage vloeistof gemeten. De resultaten zijn beschreven in **hoofdstuk 3**. We hebben gevonden dat er in de type II cellen van aan Nitrofen blootgestelde longen minder rijpe multilamellaire lichaampjes (MLBs) zijn op dag 20 en meer glycogeen op dag 22. Dit suggereert een vertraagde rijping van deze type II cellen in

hypoplastische longen in vergelijking met normale longen. De fosfolipid compositie en de hoeveelheid SP-A per mol fosfolipid in de bronchoalveolaire lavage vloeistof is hetzelfde in de controle groep en de Nitrofen groep. Kortom, de samenstelling van het extracellulaire surfactant verschilt niet tussen beide groepen.

In **hoofdstuk 4** is het ontstaan van CDH met longhypoplasie bestudeerd met behulp van de scanning electronenmicroscoop. We hebben gevonden dat de verstoring van de longontwikkeling evenredig is met de hoeveelheid lever in de borstholte. Dit betekent dat groeivertraging niet zozeer het resultaat is van longcompressie in de foetus maar eerder van competitie voor ruimte in het embryo: de snelgroeiende lever reduceert de beschikbare ruimte voor de long. Als de resterende ruimte te klein is zal longhypoplasie ontstaan.

In de volgende studie, beschreven in **hoofdstuk 5**, hebben we geprobeerd een onderscheid te maken tussen effecten van Nitrofen op de vroege longproliferatie en de vroege longdifferentiatie. Als criteria voor proliferatie hebben we genomen: lengte van het longprimordium en kleuring met proliferating cell nuclear antigen (PCNA). Als differentiatie markers hebben we antisera tegen collageen III en IV, fibronectine en laminine gebruikt. Op dag 13 bleek de vroege groei van het longprimordium vertraagd te zijn na blootstelling aan Nitrofen op dag 10. Er werden, na gebruik van de overige markers, geen andere verschillen gevonden tussen de longen uit de controle en de Nitrofen groep.

Gegevens uit de literatuur suggereren dat Nitrofen zijn teratogene werking zou kunnen uitoefenen via een verandering van de schildklierhormoon huishouding. Dat bracht ons er toe het effect van Nitrofen op de binding van schildklierhormoon aan de α_1 en de $ß_1$ vorm van de schildklierhormoon receptor te bestuderen. We vonden dat Nitrofen de binding van T_3 aan de schildklierhormoon receptor vermindert op een niet-competitieve manier. Dus, longhypoplasie bij de rat zou kunnen ontstaan door de verminderde binding van T_3 aan zijn receptor na blootstelling aan Nitrofen. Deze resultaten zijn beschreven in **hoofdstuk 6**.

Hoofdstuk 7 biedt een overzicht en bespreking van de belangrijkste resultaten die we tot nu toe hebben verkregen met behulp van het Nitrofen diermodel. Tevens worden vooruitzichten voor de kliniek en daarmee nog samenhangende onderzoeksvragen geformuleerd.

Uiteindelijk wordt in **hoofdstuk 8** de situatie bij de mens vergeleken met die bij de rat in het Nitrofen model voor congenitale hernia diaphragmatica en longhypoplasie. De conclusie is dat we nog voorzichtig moeten zijn met de extrapolatie van kennis verkregen uit het rattemodel naar de menselijke situatie, zelfs als het model zo perfect lijkt als dit Nitrofen model.

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LIST OF PUBLICATIONS

Full publications

Van der Voet GB, Brandsma AE, Heyink E, de Wolff FA: Accumulation of aluminium in rat liver: Association with constituents of the cytosol. Pharmacol Toxicol 70:173-176, 1992.

Brandsma AE, Tibboel D, Vulto IM, Egberts J, Ten Have-Opbroek AAW: Ultrastructural features of alveolar epithelial cells in the late fetal pulmonary acinus. A comparison between normal and hypoplastic lungs, using a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia. Microsc Res Techn 26:389-399, 1993.

Kluth D, Tenbrinck R, von Ekesparre M, Kangah R, Reich P, Brandsma A, Tibboel D, Lambrecht W: The natural history of congenital diaphragmatic hernia and pulmonary hypoplasia in the embryo. J Pediatr Surg 28:456-463, 1993.

Tibboel D, Tenbrinck R, Brandsma A, Bos AP, Sluiter W, Kluth D, Ten Have-Opbroek AAW, Lachmann B, Molenaar JC: Pathogenetic and functional aspects of congenital diaphragmatic hernia (CDH); an experimental study. Kachel, W et al (Ed): Interdisziplinare Probleme in der Perinatalmedizin G Braun, Fachverlage GmbH & Co, Karlsruhe, Germany 8-11, 1993.

Brandsma AE, Ten Have-Opbroek AAW, Vulto IM, Molenaar JC, Tibboel D: Alveolar epithelial composition and architecture of the late fetal pulmonary acinus: an immunocytochemical and morphometric study in a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia. Exp Lung Res 20:491-515, 1994.

Brandsma AE, Tenbrinck R, IJsselstijn H, Scheffers EC, Gaillard JLJ, Kluth D, ten Have-Opbroek AAW, Lachmann B, Tibboel D: Congenital diaphragmatic hernia: new models, new ideas. Pediatr Surg Int, 1994, in press.

Brandsma AE, Tibboel D, Vulto I, de Vijlder JJM, Ten Have-Opbroek AAW, Wiersinga WM: Inhibition of T3-receptor binding by Nitrofen. Biochim Biophys Acta 1201:266-270, 1994.

Abstracts:

Brandsma AE, Ten Have-Opbroek AAW, Vulto IM, Molenaar JC, Tibboel D: Alveolar epithelial composition and architecture in a rat model of lung hypoplasia. Am Rev Respir Dis 145 (Suppl):A126, 1992.

Brandsma AE, Ten Have-Opbroek AAW, Vulto IM, Molenaar JC, Tibboel D: Type I cell differentiation and surfactant protein SP-A reactivity in fetal rat lung. Am Rev Respir Dis 145 (Suppl):A131, 1992.

Brandsma AE, Tibboel D, Vulto IM, Egberts J, Ten Have-Opbroek AAW: Ultrastructure of alveolar epithelial cells and surfactant composition in rat lung hypoplasia. Am Rev Respir Dis 147(Suppl):A150, 1993.

Brandsma AE, Tibboel D, Vulto IM, Ten Have-Opbroek AAW, Wiersinga WM: Lung hypoplasia is induced by Nitrofen: an effect via inhibition of T3-receptor binding? Am Rev Respir Dis 147(Suppl):A415, 1993.

Brandsma AE, Piersma AH, Hofstee-Hooftman TWA, Verhoef A, ten Have-Opbroek AAW, Tibboel D: Early lung development in the rat and its perturbation by Nitrofen exposure. Pediatr Res 36:47A, 1994.