Journal of Pediatric Surgery 50 (2015) 1251–1259

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

journal homepage: www.elsevier.com/locate/jpedsurg

Lung maturity in esophageal atresia: Experimental and clinical study☆,☆☆

Ana Catarina Fragoso [a,b,c,*], Leopoldo Martinez [a,b], José Estevão-Costa [c], Juan A. Tovar [a,b]

[a] Department of Pediatric Surgery, Hospital Universitario La Paz, Madrid, Spain
[b] Department of Congenital Malformations, INGEMM and IdiPaz Research Laboratory, Madrid, Spain
[c] Faculty of Medicine, University of Porto, Porto, Portugal

ARTICLE INFO

Key words:
esophageal atresia
tracheoesophageal fistula
lung maturity
lung hypoplasia
newborn
adriamycin rat model

ABSTRACT

Introduction: Esophageal atresia and tracheoesophageal fistula (EA-TEF) survivors suffer respiratory morbidity of unclear pathogenesis. Defective lung morphogenesis has been described in the rat model. This study examined fetal lung growth and maturity in rats and patients with EA-TEF.
Methods: Pregnant rats received either adriamycin or vehicle. Control and adriamycin-exposed lungs, with and without EA-TEF, were weighed and processed for RT-PCR, DNA quantification, immunofluorescence and immunoblot analysis of *TTF1*, *VEGF*, *Sp-B*, and *α-sma*. Twenty human lungs were also processed for immunofluorescence and Alcian-blue staining.
Results: Lungs from fetuses with EA-TEF (E21) showed decreased total DNA; *FGF7* and *TTF1* mRNA expressions were upregulated at E15 and E18, respectively. Protein expression and immunofluorescent distribution of maturity markers were similar. Lungs from stillborns with EA-TEF showed decreased epithelial expression of Sp-B and VEGF whereas those from newborns tended to have less Sp-B and more VEGF and mucous glands.
Discussion: The lungs of rats with EA-TEF were hypoplastic but achieved near-normal maturity. Stillborns with EA-TEF exhibited an apparently disturbed differentiation of the airway epithelium. Newborns with EA-TEF demonstrated subtle differences in the expression of differentiation markers, and increased number of mucous glands that could influence postnatal respiratory adaptation and explain some respiratory symptoms of EA-TEF survivors.

Esophageal atresia with tracheoesophageal fistula (EA-TEF) is a congenital malformation with an incidence of 1 per 3000 live births. Nowadays, with survival approaching 95%, the focus of interest is shifting to functional and quality of life issues throughout childhood and adulthood.

Approximately fifty percent of neonates with EA-TEF have one or more additional skeletal, anal, cardiovascular or renal malformations. Respiratory malformations occur in 6% of patients [1], in 13.2% of autopsies of newborns with EA-TEF [2], and in up to 47% of VACTERL (vertebral, anal, cardiac, tracheoesophageal, renal, limbs) association patients [3,4]. The relatively low incidence and the scarce clinical relevance of some of these malformations invite to ascribe the respiratory symptoms in these children to tracheomalacia, aspiration related to impaired esophageal motility, esophageal stricture, recurrence of tracheoesophageal fistula or gastroesophageal reflux. However, upon adequate testing, up to 75% of EA-TEF survivors have abnormal pulmonary function apparently not related to either associated conditions or to postoperative sequelae or prematurity [5]. In fact, EA-TEF survivors suffer persistent unspecific respiratory symptoms (chronic cough, asthma-like wheezing, and bronchial hyperresponsiveness) that sometimes do not improve or even become more frequent with age [6–8]. Additionally mild respiratory function disorders (obstructive/restrictive) have been demonstrated in children, adolescents and adults born with EA-TEF, but their origin remains unclear [5,6,9].

The scarcity of fetal or neonatal human tissue from affected individuals prompted the development of animal models. In 1996, Diez-Pardo et al described a toxicologic model of EA-TEF/VACTERL association. After adriamycin administration to pregnant rats during the appropriate gestational days, a significant proportion of fetuses accurately reproduced the malformations of the human VACTERL association [10]. This invaluable research tool was later extended to the mouse [11].

Because EA-TEF is the consequence of an abnormal division of the foregut into esophagus and trachea, we hypothesize that the disturbance in the early embryonic development of the foregut that results in EA-TEF may also interfere with the emergence of lung buds, and consequently lung morphogenesis. This hypothesis was further supported recently by demonstration of lung hypoplasia and abnormal control of airway branching in the lungs of fetal rats with EA-TEF [12,13].

The present study aimed at gaining new insights into lung development in EA-TEF. Lung growth and biochemical maturity were assessed

☆ This study was supported in part by IdiPaz grants and by the Spanish Health Institute Carlos III (grant N. RD08/0072: Maternal, Child Health and Development Network).
☆☆ The authors declare that they do not have any competing or financial interests.
* Corresponding author at: Faculty of Medicine, University of Porto, Alameda Hernâni Monteiro, 4200–319, Porto, Portugal.
E-mail address: catarina.fragoso@gmail.com (A.C. Fragoso).

http://dx.doi.org/10.1016/j.jpedsurg.2015.06.015
0022-3468/

in rat fetuses with esophageal atresia and in lung specimens from human fetuses and newborns with this malformation.

1. Materials and methods

Approval of the institution research ethical committee was obtained for the study (HULP PI-1501). Parental consent had been required for the retention of tissue in each case.

1.1. EA-TEF rat model

Time-dated pregnant Sprague-Dawley rats (OFA Charles River Laboratories. Cerdanyola, Spain) were treated once a day from gestational day 7–9 (morning of sperm in vaginal smear was considered day 0) by intraperitoneal injection, either of 1.75 mg/Kg adriamycin (Farmiblastina Pharmacia, Madrid, Spain) or vehicle.

1.1.1. Fetal harvesting and dissection

Cesarean section was performed on E15, E18 and E21 before euthanizing the dams with intracardiac injection of potassium chloride. At the elected time endpoints, fetuses were recovered, weighed and dissected under a microscope to document the presence of EA-TEF. Lungs were harvested, weighed, photographed and fixed or snap frozen and stored at −80° until further use. Three groups of offsprings were compared: control (C, n = 66), adriamycin-treated with EA-TEF (adria EA, n = 76) and adriamycin-treated without EA-TEF (adria noEA, n = 67).

1.1.2. Lung wet/dry weight ratio

Lungs from E21 fetuses were weighed immediately after dissection to determine the wet weight and after being placed in an incubator for 72 hours, to assess the dry weight.

1.1.3. Total DNA extraction and quantification

DNA was extracted from snap-frozen tissue with DNeasy blood and tissue extraction kit (cat# 69504, Qiagen, Las Rozas, Spain), and the purified (RNAseA 100 mg/ml, cat#19101, Qiagen, Las Rozas, Spain) total DNA content was determined with a spectrophotometer (Nano Drop; Fisher Scientific, Madrid, Spain) at 260/280 μm.

1.1.4. mRNA extraction and cDNA synthesis

Total mRNA was isolated from snap-frozen lungs using High Pure RNA Tissue Kit (Roche Applied Science, Mannheim, Germany). Concentration and purity of RNAs were determined spectrophotometrically; 250 ng of total RNAs was retrotranscripted to complementary DNAs (cDNA) by reverse transcription reactions using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, CA). All cDNAs were stored at −80°, until further use.

1.1.5. Real time reverse transcriptase polymerase chain reaction (real time RT-PCR)

TTF1 and *FGF7* lung expressions were quantified in a LightCycler 480 SYBR Green I Master (Roche Applied Science, Mannheim, Germany) using the following primer for *TTF1*: forward 5' CACCTTACCAGGACACCATG and reverse 3' GCCCATGCCGCTCATATTCA and forward 5' AAGTGAAAGGGACCCAGGAG and reverse 3' GCCACAATTCCAACTGCCAC for *FGF7*. All RT-PCR reactions were run in duplicate. The RT-PCR conditions were: 95° for 5 minutes, followed by 50 cycles at 95° for 10 seconds, 60° for 10 seconds and 72° for 10 seconds. Results were normalized to the expression of the 18S. The relative mRNA levels were determined by calculating the threshold cycle for *TTF1* and *FGF7* gene using the threshold cycle method.

1.1.6. Western blot

TTF1, Sp-B, VEGF and α-smooth muscle actin protein levels were measured in homogenized lungs (E21) in cell disruption buffer (PARIS Kit, Ambion, USA). The protein content of 50 μg of tissue was measured using a protein assay kit (Pierce; BCA Protein Assay Kit, Rockford, IL, USA). Immunoblotting was performed with 12% SDS-polyacrylamide gel with the anti-TTF1 1:300 (H-190:sc13040; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), Sp-B 1:300 (H-300: sc-13978; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), VEGF 1:400 (07-1420, Merck Millipore, Germany) and α-smooth muscle actin antibody 1:200 (1A4:sc-32251; Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Values were normalized to anti-Cu/Zn superoxide-dismutase (1:1000, Stressgen, Belgium).

1.1.7. Immunofluorescence staining

Lungs from E21 were fixed overnight in 4% paraformaldehyde. After inclusion in paraffin, 5 μm sections were stained. Immunofluorescence staining was performed using standard techniques with anti-TTF1 rabbit polyclonal antibody (H-190:sc13040. Santa Cruz Biotechnologies, Santa Cruz, CA, USA) after a dilution 1:100; anti-SpB rabbit polyclonal antibody (H-300: sc-13978; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) 1:50; anti-VEGF rabbit polyclonal antibody (07-1420, Merck Millipore, Germany) 1:400 and anti-α-smooth muscle actin mouse monoclonal antibody (1A4:sc-32251; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) 1:200. Briefly, antigen recovery was performed with sodium citrate (10 mM, pH 6) in microwave for 10 minutes followed by a 2 hours incubation with 10% horse serum, 1% albumin TBS. Sections were incubated overnight at 4 °C with primary antibodies then washed and incubated with universal secondary antibody (Vectstain Universal Quick Kit; PK-8800; Vector Laboratories, Inc. Burlingame, CA, USA) for 1 hour, and at last with Streptavidin Alexa fluor 488 conjugate (S-32354, Molecular Probes, Invitrogen, Carlsbad CA, USA) for 45 minutes. The sections were mounted and the nuclei counterstained using Vectashield Mounting Medium for fluorescence with DAPI (H 1200; Vector Laboratories, Inc. Burlingame, CA, USA). Negative control slides were stained by the same procedure, with the primary antibody omitted. Images were obtained from a minimum of 6 different slides from each rat group with a Leica LMD6000 fluorescence microscope (Leica Microsystems, Germany).

1.2. Stillborn and neonatal human lung samples

Blocks of lung tissue taken at postmortem examination, between 1985 and 2012, were obtained from the pathology department of the hospital. The inclusion criteria for the EA-TEF group were: fetuses of 33 or more weeks of gestational age, with the malformation but without primary lung disease and/or bilateral renal anomalies, oligohydramnios or chromosomal anomalies. Same criteria were used for selecting the lungs for the control group except for the presence of the tracheoesophageal malformation. Twenty lung samples, 8 from stillbirth and 12 from deceased newborns were examined. The gestational age ranged from 33 weeks to 42 weeks (with 18 of >33 weeks gestation and both groups with comparable gestational ages).

1.2.1. Immunofluorescence staining

The same protocol and antibody were used to assess Sp-B immunoreactivity in the human 5 μm lung sections. For VEGF and α-smooth muscle actin a double immunofluorescence staining was carried out with the following primary antibodies: anti-VEGF rabbit polyclonal antibody (07-1420, Merck Millipore, Germany) 1:400 and anti-α-smooth muscle actin mouse monoclonal antibody (1A4:sc-32251; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) 1:100; the secondary antibodies used were Alexa Fluor 488 goat anti-rabbit (A-11034; Molecular Probes, Life Technologies, USA) and Alexa Fluor 594 rabbit anti-mouse (A-11062; Molecular Probes, Life Technologies, USA). The sections were mounted and the nuclei counterstained using Vectashield Mounting Medium for fluorescence with DAPI (H 1200; Vector Laboratories, Inc. Burlingame, CA, USA). Negative control slides were stained by the same procedure, with the primary antibody omitted. Images were obtained