Decreased Lung Fibroblast Growth Factor 18 and Elastin in Congenital Diaphragmatic Hernia and Models

Olivier Boucherat[1], Alexandra Benachi[1,2], Anne-Marie Barlier-Mur[1], Marie-Laure Franco-Montoya[1], Jelena Martinovic[3], Bernard Thébaud[4], Bernadette Chailley-Heu[1], and Jacques R. Bourbon[1]

[1] Inserm U651, Créteil, France ; Université Paris 12, Faculté de Médecine, Institut Mondor de Médecine Moléculaire, Créteil, France.

[2] Université Paris-Descartes, Faculté de Médecine ; AP-HP ; Hôpital Necker-Enfants Malades, Maternité, Paris, France.

[3] Université Paris-Descartes, Faculté de Médecine ; AP-HP ; Hôpital Necker-Enfants Malades, Service de Fœtopathologie, Paris, France.

[4] Department of Pediatrics-Division of Neonatology, University of Alberta, Edmonton, Canada.

ONLINE DATA SUPPLEMENT

MATERIAL AND METHODS

Human Lung Tissue

Human lung samples were collected post-mortem between 14 and 37 weeks (wk) of pregnancy (fetal age) either after medical terminations of pregnancy according to the French bioethics law of July 1994, or following natural death after delivery. Tissue collection was made during the autopsy, performed at parental request. Tissue samples were collected humanely according to the standardized protocol of the French Society of Fetal Pathology. Parents were informed about the procedure and issues of post-mortem study, and signed consent was obtained for all included patients. The consent form that allows human tissue samples to be collected for research purpose, which is mandatory before any autopsy, has been established by the Ethics Committee of the French Ministry of Health. The present study was undertaken with approval of the local Ethics Committee. The diagnosis of CDH was made by echography and confirmed by postmortem examination. Reduced lung weight and consistent histological appearance confirmed the diagnosis of pulmonary hypoplasia. All fetuses were of bad prognosis, and most had other malformations or associated chromosomal abnormality. Lungs from fetuses with non-pulmonary diseases were used as controls; they were histologically normal by postmortem examination, and presented no pulmonary hypoplasia. Detailed clinical data are depicted in table 1. Specimens were frozen tissue stored at -80°C and fixed tissue for histological sections.

Sheep model of CDH and tracheal occlusion

All animal experiments were performed with authorization of the French Ministry of Agriculture. Surgical procedures have been extensively described elsewhere (36). Biological samples were collected from the same animals as in previous reports (20, 27). In brief, three groups of Pre-Alp ovine fetuses (full term = 145d) were studied. The first group, designated the surgical diaphragmatic hernia (sDH) group (n=6), was obtained by creating a left-side diaphragmatic hernia in utero at 85 d of gestation. The second group, designated the sDH +

tracheal occlusion (TO) group (n=4) was obtained by creating sDH at 85d as in the first group, then TO at 120d with latex balloon via noninvasive endoscopic technique. The third group, designated the control group (n=5), consisted of twin fetuses that were sham-operated. Fetuses were retrieved by cesarean section at 139d of gestation, and lung tissue was either immediately frozen and stored at -80°C for molecular or biochemical analysis, or fixed with paraformaldehyde for inclusion and histological observations.

Nitrofen exposure in rats

The procedure has been described in detail elsewhere (33). In brief, dated pregnant Wistar rats (Charles River, Saint Germain sur l'Arbresle, France) were gavaged with the herbicide 2,4-dichlorophenyl-p-nitrophenyl ether (Nitrofen, Rohm Haas Company, Philadelphia, PA) suspended in olive oil (100mg in 1ml) on day 12 of gestation. This resulted in lung hypoplasia in all fetuses, associated with a 60 to 70% incidence of right-sided CDH. Control dams received olive oil. Vitamin A (Avibon, Rhône-Poulenc, Paris, France), 15,000 IU, was similarly gavaged on day 14 to either control or nitrofen-treated pregnant rats. On day 21, fetuses were retrieved by cesarean section under maternal pentobarbital anesthesia. Fetuses were exsanguinated, checked for the presence of CDH, and lungs were excised, cleared of surrounding tissues, weighed, frozen in liquid nitrogen, then stored at -80°C until processing.

Histochemical elastin staining and quantification

Because of restrictions of human tissue sampling conditions, and of collection of sheep lung samples for multiple purposes including electron microscopy, biochemical, and molecular biology analyses (20, 27), it was not possible to proceed to lung fixation at constant pressure, and therefore to perform morphometry. Pieces of human lung tissue were fixed in formalin 24h after death; pieces of sheep lung tissues were fixed in paraformaldehyde 4% at sacrifice. Fixed tissues were paraffin-embedded. Five µm sections were dehydrated in xylene and graded ethanol, and then stained with Weigert's stain for elastin. Light microscopy images

were captured with a digital camera. The proportion of tissue surface-area occupied by elastic fiber deposits was determined with Perfect Image v7.4 software according to the manufacturer's instructions. In each analyzed field, parenchymal tissue area was determined by subtracting airspace surface-area from total surface-area, and stained elastic fiber surface-area was measured after exclusion of large vessel and airway elastin.

Immunohistochemical FGF18 analyses

Sections were deparaffinized, rehydrated, and boiled in 0.1M sodium citrate for 15 mn. After blocking with 1% BSA in PBS plus 5% normal donkey serum, sections were labeled for FGF18 using a polyclonal antibody raised in rabbit (AbCys S.A., Paris, France). A Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, Newmarket, UK) was used as the secondary antibody. Sections incubated either with PBS/BSA, or pre-immune, or non-immune rabbit serum instead of the primary antibody, were used as negative labeling controls. Sections were mounted using Vectashield medium with DAPI (VECTOR Laboratories, Burlingame, CA) to visualize nuclei, and photographed using a Zeiss microscope equipped with a digital camera (KAPPA Opto-electronics, Gleichen, Germany).

RNA extraction

Total RNAs were extracted from lung tissue using Trizol reagent (Invitrogen, Cergy-Pontoise, France) according to the manufacturer's instructions. The pelleted RNA was dissolved in sterile water and quantified by absorbance at 260nm with a spectrophotometer (Biophotometer Eppendorf, Wesseling-Berzdorf, Germany). Quality and integrity were confirmed after electrophoresis of 1µg of each sample in 1% agarose gel.

Determination of ovine partial cDNA sequence for FGF18

cDNAs were reverse-transcribed from sheep lung total RNAs with Superscript II reverse transcriptase and random hexamer primers (Invitrogen) according to the manufacturer's instructions. Amplification of partial cDNA sequence for FGF18 was performed using sense

primer 5'- CTGCTGTGCTTCCAGGTTCA -3' (mouse/rat FGF18-specific sequence, GenBank accession numbers NM008005 and NM019199, respectively) and antisense primer: 5'-CCGTCGTGTACTTGAAGGGC -3' (human FGF18-specific sequence, GenBank accession # BC006245). Amplification was performed through 35 cycles (1 min at 94°C, 1 min at 59°C, and 1 min at 72°C). The amplified sequence was purified with QIAquick PCR purification kit (Qiagen, Courtaboeuf, France) before being sequenced with ABI 3130 XL gene analyzer (Applied Byosystems, Courtaboeuf, France).

Northern blot analysis

Twenty micrograms of RNAs were fractionated by electrophoresis through 1.2% agarose, 2.2M formaldehyde gels, then transferred onto nylon membranes (Perkin Elmer Life Sciences, Courtaboeuf, France) by capillarity. Rat cDNA probes consisted of a 1,100-bp sequence for tropoelastin (gift from Dr. C. Rich, Philadelphia, PA) and a 904-bp sequence for FGF18 (gift from Dr. N. Itoh, Kyoto, Japan). Ovine tropoelastin cDNA probe was obtained by RT-PCR from RNAs extracted from fetal sheep lung tissue, using ovine-specific oligonucleotide primers (37). Probes were labeled with (α^{32}P) dCTP (Perkin Elmer Life Sciences) using the Rediprime labelling system (Amersham Biosciences, Orsay, France), and purified on G-50 probe-purification columns (Amersham Biosciences). Membranes were pre-incubated and probed overnight at 42°C in hybridization buffer containing 50% formamide, 50mM Tris-HCl (pH 7.5), 0.8M NaCl, 10% dextran sulfate, 0.1% sodium pyrophosphate, 5X Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS) and 75µg/ml denatured salmon sperm. Membranes were successively probed for tropoelastin and/or FGF18 mRNA, then 18S rRNA. Blots were exposed to X-Omat AR Kodak films for a suitable exposure time at -80°C. Autoradiographic signals were quantified by densitometry using image analysis software (NIH Image, Bethesda, MD) and normalized to the relative amount of 18S rRNA.

Reverse Transcription and Real-time quantitative PCR

RNAs were reverse transcribed into cDNA using 2 µg of total RNA, Superscript II reverse transcriptase and random hexamer primers (Invitrogen) according to the manufacturer's protocol. Real time PCR was carried out to determine semi-quantitatively the amounts of FGF18 mRNA, FGFR3 mRNA, and internal reference 18S rRNA in ovine lungs. Primer sequences designed using Primer Express software (Applied Biosystems) are reported in table 2. Relative levels of gene expression were determined using SYBR Green master mix (Applied Byosystems) according to the manufacturer's instructions, and ABI PRISM® 7000 Sequence Detection System (Applied Byosystems) with the following program: 10 min 95°C, (15 sec 95°C, 1 min 60°C) x40. Melt curve analysis was performed to confirm that a single specific amplified product was generated. Relative expression was determined by using the $\Delta\Delta$Ct (threshold cycle) method of normalized samples (ΔCt) in relation to the expression of a calibrator sample, according to the manufacturer's protocol. Samples without reverse transcriptase (non-RT samples) were used as negative controls for real-time PCR. All measurements were performed in triplicates.

Western blot analysis

Lung tissue was homogenized in RIPA buffer containing protease inhibitors cocktail (Roche Diagnostics, Mannheim, Germany). Homogenates were centrifuged 10 min at 10,000x*g*, supernatants were collected and protein concentration was determined by the Bradford assay method. Forty or 60 µg of proteins were electrophoresed on a 8 to 12% SDS-polyacrylamide gel, then transferred onto a polyvinylidene fluoride membrane (Millipore, Saint-Quentin en Yvelines, France); transfer was checked by stain with Ponceau S dye (Sigma, L'Isle d'Abeau, France). Membranes were blocked with 5% nonfat dry milk in Tris-buffer saline containing 0.05% tween-20 (TTBS) at room temperature for 2h. Antibodies were diluted in 2% nonfat dry milk in TTBS. Membranes were exposed for 2h to a goat anti-rhFGF18 antibody (R&D Systems, Lille, France) diluted 1:500, washed in TTBS, then incubated for 1h with horseradish peroxidase-conjugated donkey anti-goat IgG antibody

(Santa Cruz Biotechnology, Santa Cruz, USA) diluted 1:5000, respectively. After washing in TTBS, membranes were incubated for 1 min in Enhanced Chemiluminescence detection reagent (Amersham Biosciences), before exposure to Kodak BioMax MS film for 2 min. Densitometry analysis of blots was performed using NIH image software.

Statistical analysis

Data are presented as mean ± se. Multiple group comparisons were made either by ANOVA followed by Fisher's PLSD, or by non-parametric Kruskall-Wallis analysis, depending on applicability as detailed in results. Two-group comparisons were made by Student's *t* test or by non-parametric Man and Whitney's U test, depending on applicability. P=0.05 was considered as the limit of statistical significance.