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Prenatal retinoic acid increases lipofibroblast expression in hypoplastic rat lungs with experimental congenital diaphragmatic hernia

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ABSTRACT

Background/purpose: Prenatal administration of all-*trans* retinoic acid (ATRA) has been shown to stimulate alveolarization in nitrofen-induced pulmonary hypoplasia (PH) associated with congenital diaphragmatic hernia (CDH). Lipid-containing interstitial lipofibroblasts (LIFs), characterized by adipocyte differentiation-related protein (ADRP), play a critical role in alveolar development by coordinating lipid homeostasis. Previous studies have demonstrated that ATRA positively affects LIF expression in developing lungs. We hypothesized that pulmonary LIF expression is increased after prenatal ATRA treatment in the nitrofen model of CDH-associated PH.

Methods: Timed-pregnant rats were treated with nitrofen or vehicle on E9.5, followed by injection of ATRA or placebo on E18.5, E19.5, and E20.5. Fetal lungs were dissected on E21.5 and divided into Control + Placebo, Control + ATRA, Nitrofen + Placebo, and Nitrofen + ATRA. Pulmonary gene expression levels of *ADRP* were analyzed by quantitative real-time polymerase chain reaction, and LIF expression was investigated by ADRP immunohistochemistry, oil-red-O-, and immunofluorescence-double-staining.

Results: Relative mRNA expression of pulmonary *ADRP* was significantly increased in Nitrofen + ATRA compared to Nitrofen + Placebo (0.31 ± 0.02 *vs.* 0.08 ± 0.01; $P < 0.0001$). ADRP immunoreactivity and oil-red-O-staining were markedly increased in alveolar interstitium of Nitrofen + ATRA compared to Nitrofen + Placebo. Immunofluorescence-double-staining confirmed markedly increased LIF expression in alveolar walls of Nitrofen + ATRA compared to Nitrofen + Placebo.

Conclusions: Increased LIF expression after prenatal treatment with ATRA in nitrofen-induced PH suggests that ATRA may have a therapeutic potential in attenuating CDH-associated PH by stimulating alveolar development.

Congenital diaphragmatic hernia (CDH), currently affecting about 2.5 per 10,000 newborns [1], remains one of the major therapeutic challenges in neonatal intensive care. Overall, neonatal mortality and long-term morbidity continue to be relatively high among CDH patients despite modern treatment with exogenous surfactant, inhaled nitric oxide and extracorporeal membrane oxygenation [2]. Together with persistent pulmonary hypertension, pulmonary hypoplasia (PH), which is characterized by immature lung development and impaired alveolarization, is considered to be the main reason for the unsatisfactory survival rate [3]. Most of our current understanding about the structural and molecular changes in hypoplastic lungs originated from experimental animal models [4]. The nitrofen rodent model of CDH has widely been used to investigate the pathogenesis of PH, as it reproduces diaphragmatic defects and bilateral PH remarkably similar to the human situation [5].

All-*trans* retinoic acid (ATRA), the active metabolite of vitamin A, is an essential component of the gene network that regulates fetal growth of several organ systems, including lung morphogenesis and diaphragmatic development [6]. Recent results from various animal research have indicated that disruption of retinoid signaling contributes to the formation of CDH [7,8]. It has further been demonstrated that prenatal administration of ATRA upregulates pulmonary expression of several genes involved in the retinoic acid signaling pathway [9]. In addition, it has been shown that ATRA rescues PH in nitrofen-induced hypoplastic lung explants [10]. In vivo studies in rats with CDH have provided additional evidence that prenatal administration of ATRA stimulates alveolarization in nitrofen-induced hypoplastic lungs [11], suggesting a therapeutic potential of ATRA in attenuating CDH-associated PH. However, the exact molecular and cellular effects of ATRA treatment on fetal alveolar development remain unclear.

Lipid-containing interstitial lipofibroblasts (LIFs) play a critical role in alveolar development by coordinating lipid homeostasis and *de novo* synthesis of surfactant phospholipids [12,13]. In rodents, LIFs are

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first evident during the late canalicular phase of lung development with a significant increase over the last few days of gestation [14,15]. Adipocyte differentiation-related protein (ADRP), a lipogenic molecular marker characterizing LIFs, has been found to be highly expressed in fetal lungs [16,17] with an increase immediately before birth [18]. Recent work from our laboratory has shown that the expression of LIFs and thus ADRP is significantly decreased in CDH-associated PH, suggesting a clear disturbance of normal LIF functioning in hypoplastic lungs [19]. Moreover, previous studies have demonstrated that ATRA positively affects differentiation and proliferation of LIFs during fetal lung development [20], suggesting a strong therapeutic potential of ATRA for the treatment of alveolar immaturity in PH. We designed this study to investigate the hypothesis that prenatal administration of ATRA increases pulmonary LIF expression in the nitrofen rat model of CDH-associated PH.

1. Material and methods

1.1. Animals, drugs and experimental design

After obtaining ethical approval (Ref. REC668b) from the Research Ethics Committee, adult *Sprague–Dawley*® rats (Harlan Laboratories, Shardlow, UK) were mated overnight and females were checked daily for presence of vaginal plug. The day of plugging was defined as embryonic day 0.5 (E0.5) and timed-pregnant animals were randomly divided into two groups ("Nitrofen" and "Control"). On E9.5, dams were briefly anesthetized with 2% volatile isoflurane (Piramal Healthcare Ltd, Morpeth, UK) and 100 mg nitrofen (2,4-dichloro-4′-nitrophenylether) (Wako Chemicals GmbH, Neuss, Germany) was administered in 1 ml olive oil via oral-gastric lavage, while control animals received vehicle alone. Five milligram/kg ATRA (Sigma Aldrich, Saint Louis, USA) was dissolved in 1 ml cottonseed oil and injected intraperitoneally under short anesthesia on E18.5, E19.5 and E20.5, while control rats received dissolvent alone. On the selected end point E21.5, fetuses were delivered via caesarean section and sacrificed by decapitation. After laparotomy, fetuses were inspected for diaphragmatic defects and dissected left lungs were divided into four experimental groups: Control + Placebo ($n = 16$), Control + ATRA ($n = 16$), Nitrofen + Placebo ($n = 16$) and Nitrofen + ATRA ($n = 16$). Eight lungs from each experimental group were snap-frozen in liquid nitrogen (and stored at −80 °C) for RNA isolation and subsequent quantitative real-time polymerase chain reaction (qRT-PCR), while 8 specimens were fixed in 4% paraformaldehyde (PFA) (Santa Cruz Biotechnology Inc, Santa Cruz, USA) for 24 h until further processing for immunohistochemical/immunofluorescence and oil-red-O-staining was carried out. In total, 64 fetal animals from 8 different dams were used in this study.

All animal procedures were performed following current guidelines for management and welfare of laboratory animals and were approved by the Department of Health and Children (Ref. B100/4378) under the Cruelty to Animals Act, 1876 (as amended by European Communities Regulations 2002 and 2005), in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

1.2. Total RNA isolation and complementary DNA synthesis

Total RNA was isolated from snap-frozen left lungs with the acid guanidinium thiocyanate-phenol-chloroform extraction method using a TRIzol® reagent (Invitrogen™ by Life Technologies™, Carlsbad, USA) according to the manufacturer's protocol. Concentration and purity of total RNAs were determined spectrophotometrically using a NanoDrop ND-1000 UV–vis® (Thermo Scientific Fisher, Wilmington, USA). One microgram of total RNA was retroscripted to complementary DNA (cDNA) by reverse transcription reaction using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. All cDNA samples were stored at 4 °C until further use.

1.3. Quantitative real-time polymerase chain reaction

ADRP mRNA expression was quantified with a LightCyler® 480 system using a SYBR® Green I Master Mix (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. qRT-PCR conditions were 95 °C for 5 min, followed by 55 amplification cycles at 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 10 s. Gene-specific primer sequences used for *ADRP* were 5′-CTCTCGGCAGGATCAAAGAC-3′ and 5′-CGTAGCCGACGATTCTCTTC-3′. Relative mRNA expression levels were determined using the comparative cycle threshold method. Results were normalized to the expression of our housekeeping gene *β-actin* (5′-TTGCTGACAGGATGCAGAAG-3′ and 5′-TAGAGCCACCAATCCACACA-3′). All qRT-PCR experiments were run in duplicate for each sample.

1.4. ADRP immunohistochemical staining

After fixation in PFA, left lungs were paraffin-embedded and cut transversely at a thickness of 5 μm. Tissue sections were deparaffinized in xylene, followed by gradual rehydration in ethanol and distilled water. Sections were incubated with phosphate buffered saline (PBS) (Oxide Ltd, Basingstoke, UK) containing 1.0% Triton X-100 (Sigma Aldrich, Saint Louis, USA) for 20 min to improve cell permeabilization. In order to avoid masking of antigenic sites, sections were immersed in heated Target Retrieval Solution® (DAKO Ltd, Cambridgeshire, UK) in a microwave oven at 750 W for 15 min and endogenous peroxidase activity was blocked using a Peroxidase Block® (DAKO Ltd, Cambridgeshire, UK) for 5 min according to the manufacturer's protocol. To prevent nonspecific absorption, sections were blocked with 10% normal goat serum (Sigma Aldrich, Saint Louis, USA) for 30 min, followed by overnight incubation with affinity-purified rabbit ADRP antibodies (sc-32888, 1:50) (Santa Cruz Biotechnology Inc, Santa Cruz, USA) at 4 °C. On the next day, sections were washed in PBS + 0.05% Tween and incubated with horseradish peroxidase-conjugated antirabbit secondary antibodies (K4011, 1:100) (DAKO Ltd, Cambridgeshire, UK). Antibody–antigen complexes were visualized by staining with diaminobenzidine (DAB) + Substrate Buffer® and DAB + Chromogen® (DAKO Ltd, Cambridgeshire, UK) for 30 s. After counterstaining with hematoxylin (Sigma Aldrich, Saint Louis, USA) for 10 s, sections were mounted with glass coverslips using DPX Mountant for histology (Sigma Aldrich, Saint Louis, USA). All sections were independently evaluated by two investigators with a Leica DM LB research microscope (Leica Microsystems GmbH, Wetzlar, Germany).

1.5. Oil-red-O-staining

Formalin-fixed left lungs were washed overnight in PBS, followed by embedding in O.C.T. Compound Mounting Medium (VWR International Ltd, Dublin, Ireland) and freezing at −80 °C. Frozen blocks were cut transversely at a thickness of 10 μm and tissue sections were mounted on SuperFrost Plus slides (VWR International Ltd, Dublin, Ireland). Air-dried, thawed sections were rinsed three times with distilled water and immersed in 100% propylene glycol (Sigma Aldrich, Saint Louis, USA) for 5 min. Oil-red-O-solution was prepared by slowly dissolving 0.7 g oil-red-O-powder (Sigma Aldrich, Saint Louis, USA) in 100 ml propylene glycol, while heating to 100 °C for a few minutes. This solution was filtered twice and cooled down before further use. Sections were immersed in oil-red-O-solution for 7 min, followed by 3 min in 85% propylene glycol. After counterstaining with hematoxylin (Sigma Aldrich, Saint Louis, USA) for 30 s, sections were mounted with glass coverslips using Mowiol® (Sigma Aldrich, Saint Louis, USA) and independently evaluated by two