Improvement of pulmonary hypoplasia associated with congenital diaphragmatic hernia by in utero CFTR gene therapy

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Larson, Janet E., and J. Craig Cohen. Improvement of pulmonary hypoplasia associated with congenital diaphragmatic hernia by in utero CFTR gene therapy. Am J Physiol Lung Cell Mol Physiol 291: L4–L10, 2006. First published February 10, 2006; doi:10.1152/ajplung.00372.2005.—Congenital diaphragmatic hernia (CDH) may be an ideal candidate disease for in utero gene therapy as disrupted fetal lung growth plays a significant role in disease outcome. We previously demonstrated that transient in utero overexpression of CFTR during fetal development resulted in lung epithelial proliferation and differentiation. We hypothesized that gene therapy with CFTR would improve the pulmonary hypoplasia associated with congenital diaphragmatic hernia (CDH). CDH was induced by the herbicide 2,4-dichlorophenyl-4-nitrophyl ether (nitrofen) following maternal ingestion at either 10 or 13 days gestation. In utero gene transfer of the CFTR gene was subsequently performed at 16 days gestation. Examination of the fetuses at 22 days gestation revealed little improvement in the CFTR-treated lungs following induction of hernias with nitrofen at 10 days gestation. However, the CFTR gene treatment significantly improved internal surface area, saccular density, overall saccular number, and amount of saccular air space in the lungs that were treated with nitrofen at 13 days gestation. RT-PCR demonstrated that gene transfer occurred following treatment at 13 days gestation but not in the lungs treated with nitrofen at 10 days gestation, despite gene transfer at the same gestational age (16 days) in both groups. As disruption of lung development correlates with the gestational stage at which nitrogen exposure occurs, these results confirmed previous findings that in utero gene transfer efficiency depends on the stage of lung development. Lung development may be significantly delayed in human CDH to allow for successful gene transfer later in gestation, providing a substantial therapeutic window.

Cystic fibrosis transmembrane conductance regulator; lung development

CONGENITAL DIAPHRAGMATIC HERNIA (CDH) occurs in 1 of every 2,000–4,000 births. The clinical course of these infants is complicated by severe respiratory failure and persistent pulmonary hypertension. Despite aggressive management, the mortality and morbidity in these infants remain high (16).

The outcome of CDH is complicated by the degree of associated pulmonary hypoplasia (12, 14), and infants often succumb to respiratory failure despite physical repair of the hernia. In both human disease and animal models of CDH the disrupted lung growth is associated with epithelial cell immaturity (1, 10, 11, 17, 21). Many of these children are diagnosed antenatally, providing the option for prenatal treatment aimed at improving lung growth. Experimental interventions aimed at reducing the developmental arrest and pulmonary hypoplasia include antenatal steroids (9), tracheal occlusion (6), and prenatal vitamins A (2, 18, 19), C (14), and E (3).

A primary area of expertise in this laboratory is the intrauterine transfer of genes into the pulmonary epithelium via the amniotic fluid (15). We have previously demonstrated that transient in utero overexpression of cftr during prenatal fetal development results in lung epithelial proliferation and accelerated differentiation in the rat, mouse, and nonhuman primate (5, 7, 8). We reasoned that CDH might be an ideal candidate for in utero gene therapy because disrupted fetal lung growth plays a significant role in the poor outcome of the disease. Our aim was not to correct the hernia, but rather improve the pulmonary hypoplasia associated with CDH. We hypothesized that in utero gene therapy with CFTR would achieve this.

This hypothesis was tested in the 2,4-dichlorophenyl-4-nitrophyl ether (nitrofen)-induced fetal rat model of CDH. CDH is induced by the herbicide nitrofen following maternal ingestion at day 10 or 13 of gestation (positive vaginal smear = day 1). Dams treated with nitrofen at 10 days gestation have fetuses that develop predominantly left-sided hernias, and those treated with nitrofen at 13 days gestation have fetuses with predominantly right-sided hernias (2). Littersmates that do not have diaphragmatic hernias also have pulmonary hypoplasia that is intermediate compared with CDH and controls (4). We examined the effects of in utero cftr gene transfer on lung growth of nitrofen-treated fetuses treated at either 10 or 13 days gestation.

EXPERIMENTAL PROCEDURES

Nitrofen CDH Model

Time-dated pregnant Sprague-Dawley rats were housed under standard vivarium conditions. Fetal CDH was induced by orally administering 100 mg of nitrofen (Wako Bioproducts, Richmond, VA) dissolved in 2 ml of olive oil via oral gastric tube following brief isoflurane anesthesia. Protocol I treated time-dated pregnant Sprague-Dawley rats at 10 days gestation to induce left-sided diaphragmatic hernias followed by in utero gene transfer at 16 days gestation. Protocol II treated time-dated pregnant Sprague-Dawley rats at 13 days gestation to induce right-sided diaphragmatic hernias followed by in utero gene transfer at 16 days gestation (positive vaginal smear = day 1; gestation 22 days). Control animals received 2 ml of olive oil followed by in utero gene transfer at 16 days gestation. The experimental protocols are summarized in Fig. 1.

In Utero Gene Transfer

The fetuses were treated with gene therapy at 16 days gestation with either Ad.CMVEGFP or Av1CF2. This time point has been previously determined by our laboratory to result in the highest lung gene transfer in the rat following intra-amniotic injection (15). Gene transfer before day 16 in the rat results in low (15 days gestation) or...
Fig. 1. Experimental design. The experimental protocols did not vary with the exception of timing of nitrofen treatment. Congenital diaphragmatic hernia (CDH) was induced by nitrofen following maternal ingestion at day 10 or 13 of gestation (positive vaginal smear = day 1). Both groups received gene therapy at 16 days gestation, shown previously by our laboratory to be the ideal time for pulmonary gene transfer in the rat. Both groups were analyzed for gene transfer efficiency and pulmonary changes at 22 days gestation, immediately before birth.

no gene transfer (<14 days gestation). Gene transfer at >18 days gestation results in an immune response to the adenovirus vector and not gene transfer. Thus, in the rat, effective gene transfer to the lung can only be accomplished technically efficiently between days 16 and 18 gestation. AdCMVEGFP expresses the reporter green fluorescent protein and was used as a control for injections and a confirmation of transfection efficiency. Av1CF2 is the replication-defective adenovirus that expresses human CFTR. After induction with 5% isoflurane in a chamber and under continued inhaled sedation with 2% isoflurane with a nose cone, the abdomen was cleansed and a laparotomy was performed. A midline vertical incision was made in the abdominal wall followed by a second vertical incision into the peritoneal cavity exposing the uterine horns. Each horn was individually exposed, and the individual amniotic sacs of the fetuses were visualized. The sacs are injected with a fine gauge needle (27) and resealed almost immediately. A concentration of 10^9 pfu/ml of vector in DMEM was injected into the individual fetal sacs at a volume of 10% of the amniotic fluid volume. This resulted in a final amniotic fluid concentration of 10^9 pfu/ml. This ensured that all of the experimental animals were controlled for surgical manipulation, vector injection, and the 10% increase in amniotic fluid volume that occurs with gene transfer. In addition, one group received olive oil followed by intrauterine control virus [enhanced green fluorescence protein (EGFP)], and another group received olive oil followed by virus containing cfr. These two groups served as additional controls. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

Evaluation of Survival and Hernia Incidence

The fetuses were delivered by caesarean section at 22 days gestation following maternal anesthesia to prevent fetal breathing. The presence of a diaphragmatic hernia was determined by probing for a diaphragmatic defect from the abdomen to the thorax (19). Fetuses were randomly assigned for biochemical or morphometric analysis. Survival was evaluated by visual inspection of the fetus for evidence of resorption including small size, bone and tissue hypertrophy, and fetal degeneration.

Morphometric Analysis

After determination of hernia status, the trachea was cannulated with a 24-gauge catheter. The thoracic cavity was widely opened, and the lungs were inflated in situ at a constant pressure at 20 cmH2O for 24 h in methanol-free 4% buffered parafomaldehyde. Lungs that did not maintain constant inflation were eliminated from the analysis. The presence or absence of a hernia was confirmed by a separate observer after fixed inflation. Total fixed lung volumes were determined by water displacement (13), and the left and right lungs were embedded separately in paraffin for individual analysis. Tissues were coded and identified by a number that each fetus received at the time of death. This code was used for identification of all histological samples. Two investigators performed morphometry by using the identification numbers with treatment groups unidentified. The right and left lungs were evaluated separately. Twenty images from each lung were captured at ×200 for point-counting morphometry.

Volume Proportion of Tissues

Volume densities of saccular air space and parenchyma, airway air space and airway wall, and vessels were estimated by point-counting morphometry. After image capture, sections were counted using a lattice of 110 test points. Parenchyma was defined as the gas-exchanging compartment that contained the air spaces (saccule ducts and saccules). Airways consisted of conducting airways to the level of the terminal bronchioles.

Measurement of Lung Complexity

The complexity of the lung was determined by saccular (future air space) counts and internal surface area (20). The number of saccules was counted at ×200 final magnification per known unit area. The number of saccules per unit volume (Nv) was determined by the formula:

\[ N_v = \frac{J}{\beta \cdot \text{volume proportion of saccules}} \]

where J is the distribution constant set to 1.0 and β is the shape constant set to 1.55, the Weibel and Gomez shape constant (20).

The total number of saccules was then calculated by multiplying the number of saccules per unit volume by the volume of the lung. Interair-space wall difference [mean linear intercept (Lm)] was determined by counting the number of intercepts over a line of known length/2 at ×200 final magnification. The Lm and the lung volume (VL) were then used to calculate the surface area of the gas-exchange region of the lung using the formula: 4VL/Lm.

Real-time PCR for CFTR mRNA

Tissues were placed in RNA Later (Qiagen) at 4°C before extraction of total RNA using the Qiagen Total RNA extraction kit. Total RNA was quantitated from the optical density at 260 nm, and cDNA synthesis was performed. Primers were generated for the target gene

Table 1. Survival and incidence of hernias at 22 days gestation

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<td>Number treated</td>
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<td>109</td>
<td>62</td>
<td>52</td>
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<tr>
<td>Number survived (%)</td>
<td>31 (33)</td>
<td>46 (42)</td>
<td>48 (77)</td>
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<td>Number hernias in survivors (%)</td>
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<td>24 (52)</td>
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Survival and incidence of hernias in nitrofen-treated animals following enhanced green fluorescence protein (EGFP) or CFTR gene transfer. Fetuses treated at 13 days gestation with nitrofen had marked improvement of survival at 22 days gestation.
CFTR gene therapy in CDH

(CFTR) by SuperArray Bioscience; these primers, as well as those for the reference gene, were designed for maximum PCR efficiency. The comparative threshold cycle (Ct) method, also known as the ΔΔCt method, where

\[ \Delta \Delta C_t = \Delta C_{\text{sample}} - \Delta C_{\text{control}} \]

Sample difference = \(2^{-\Delta \Delta C_t}\)

was used. ΔCT sample was the Ct value for any sample normalized to the endogenous housekeeping gene, and ΔCT control was the Ct value for the calibrator also normalized to the endogenous housekeeping gene.

Statistical Analysis

Left and right lungs were analyzed separately. No significant differences were detected between the left and the right lung within any treatment group as determined by the paired t-test; therefore, data from both lungs were pooled within each treatment. \(\chi^2\) analysis was performed on the incidence of survival and hernia. One-way analysis of variance was used for statistical analysis followed by Dunnett’s posttest for comparison of control-EGFP animals to all treatment groups. Separate comparisons were made between the nitrofen-treated groups that developed hernias and the nitrofen-treated groups that did not develop hernias by unpaired t-tests. A P value of < 0.05 was considered significant. All values are presented as means ± SE.

RESULTS

A total of 93 fetuses were injected with control virus following nitrofen treatment, and 109 fetuses were injected with virus encoding cftr following nitrofen treatment at 10 days gestation. After nitrofen treatment at 13 days gestation, a total of 61 fetuses were injected with control virus, and 52 fetuses were injected with virus encoding cftr virus. In addition, 58 fetuses received control virus, and 61 received virus encoding cftr following ingestion of olive oil without nitrofen.

Intrauterine injections by direct visualization at 16 days gestation afforded the opportunity to evaluate late-gestation fetal demise with nitrofen treatment. A significant portion of the fetuses that received nitrofen followed by gene transfer at 16 days gestation were resorbed or dead at the time of caesarean section at 22 days gestation. As shown in Table 1, nitrofen treatment at 13 days gestation resulted in an overall lower mortality than at 10 days gestation. The difference in mortality rate between the two groups reflected the effects of nitrofen teratogenicity and the gestations at which the fetuses were exposed. The control animals injected with the EGFP control virus and that did not receive nitrofen had an 88% survival rate.

Ten-Day Nitrofen Treatment Group

Morphometrics. As previously reported (2), fetuses treated at 10 days gestation developed almost exclusively left-sided hernias. The fetuses treated with nitrofen-EGFP had a 61% incidence of hernias, and the fetuses treated with nitrofen-

Fig. 2. Morphometry of air-exchanging parameters in lungs following nitrofen treatment at 10 days gestation. The lung volumes (A), internal surface area (B), and sacculles per area (C) in the nitrofen-CFTR-treated groups did not significantly differ from their nitrofen-EGFP-treated counterparts following treatment at 10 days gestation [\(\ast P < 0.05\) difference with control-enhanced green fluorescence protein (EGFP) group]. Morphometry was performed using identification numbers with the treatment groups unidentified. Two investigators blinded to the treatment groups performed the analysis.
CFTR had a 52% incidence of hernias. This trend did not reach statistical significance.

The body weights at 22 days gestation in all of the groups exposed to nitrofen at 10 days gestation were significantly lower than the control-EGFP group (\(*P < 0.001\)). CFTR treatment did not affect body weight in these experimental groups. The control fetuses weighed 6.14 ± 0.19 g, and the nitrofen-EGFP-CDH, nitrofen-EGFP-no CDH, nitrofen-CFTR-CDH, and nitrofen-CFTR-no CDH weighed 3.54 ± 0.33, 4.67 ± 0.21, 3.98 ± 0.28, and 4.0 ± 0.25 g, respectively. This decrease in body weight of the fetuses treated with nitrofen is consistent with previous reports and reflected the general teratogenicity of the herbicide (6).

**Lung complexity.** LUNG VOLUMES. The data reflective of the lung complexity in the 10 day-nitrofen treatment group are summarized in Fig. 2. The specific fixed lung volumes of all nitrofen 10-day treatment groups were significantly smaller than the control-EGFP lung volumes and shown in Fig. 2A. Treatment with CFTR did not significantly improve the lung volumes in this experimental group. The two parameters that reflect the complexity of the air-exchanging portion of the lung are gas-exchanging surface area and number of saccules per lung. These data are detailed below and shown in Fig. 2, B and C.

**Internal gas-exchanging surface area.** The interair-space wall difference (Lm) did not differ between the nitrofen-treated CDH or non-CDH groups. The internal surface area of the gas-exchanging portion of the lung, which is a function of the Lm and the lung volume, was significantly smaller in all of the nitrofen-treated groups compared with the controls. This is demonstrated in Fig. 2B.

**Total saccules.** The total specific saccular number of the groups is shown in Fig. 2C. The saccular number was decreased in all of the nitrofen-treated animals, though compared with the control only the nitrofen-EGFP-CDH animals varied significantly from the controls.

**Thirteen-Day Nitrofen Treatment Group**

**Morphometrics.** Nitrofen treatment at 13 days gestation resulted in an overall lower mortality than treatment at 10 days gestation (Table 1). The survival rate of the fetuses treated with nitrofen followed by EGFP did not statistically differ from the survival rate of the fetuses treated with nitrofen followed by CFTR. There was a trend toward statistical improvement in the incidence of hernia between the groups, but this did not reach statistical significance (68.7% in the nitrofen-EGFP-treated group and 55.5% in the nitrofen-CFTR-treated group; \(*P = 0.1\)).

The body weights, while generally increased over those treated with nitrofen at 10 days, were still smaller following nitrofen treatment at 13 days compared with the control-EGFP group (\(*P < 0.001\)). CFTR did not significantly affect this
parameter. The control group weighed 6.14 ± 0.19 g, and the nitrofen-EGFP-CDH, nitrofen EGFP-no CDH, nitrofen-CFTR-CDH, and nitrofen-CFTR-no CDH weighed 4.61 ± 0.18, 4.84 ± 0.18, 4.99 ± 0.16, and 5.08 ± 0.06 g, respectively.

**Lung complexity. LUNG VOLUMES.** In contrast to the fetuses treated with nitrofen at 10 days, there was marked improvement in the fetal lungs that received the CFTR gene following nitrofen treatment at 13 days. These data are summarized in Fig. 3. There was an increase in the specific lung volumes in the CFTR treatment groups with the difference reaching statistical significance between the nitrofen non-CDH groups (Fig. 3A). The parameters reflective of air-exchanging portion of the lung significantly improved in the nitrofen-CFTR-treated animals compared with the nitrofen-EGFP animals. These were the gas-exchanging surface area and number of saccules per lung. These data are detailed below and summarized in Fig. 3, B and C.

**INTERNAL SURFACE AREA.** The interair-space wall difference (Lm) was decreased in the nitrofen-CFTR treated lungs and comparable to controls. The decreased Lm reflected a more complex air-exchanging unit. The internal surface area of the gas-exchanging portion of the lung, which is a function of the Lm and the lung volume, was significantly improved in the nitrofen-CFTR-treated lungs. Both the CDH and non-CDH CFTR-treated groups varied significantly from their respective EGFP-treated groups (Fig. 3B).

**TOTAL SACCULES.** In addition to their increased lung volumes the nitrofen-CFTR treated fetuses had increased numbers of saccules per unit area of lung. Both groups of nitrofen-CFTR treated animals had significantly higher numbers of saccules/cm² compared with their respective nitrofen-EGFP groups. This was in both the CDH and non-CDH groups. Larger lungs with more saccules/area resulted in significantly increased numbers of saccules in the lungs of both of the nitrofen-CFTR treated groups. As shown in Fig. 3C, the specific number of saccules in the nitrofen-CFTR groups approached normal.

**Lung histology.** The histological appearance between the 13 day-treated nitrofen-EGFP and nitrofen-CFTR groups was markedly different. This histology reflected the increased air-exchanging area in the CFTR-treated lungs. The animals treated with nitrofen-EGFP animals with CDH (Fig. 4A) had the markedly thick interstitium and compressed air spaces seen previously with CDH and indicative of developmental arrest (4). This is caused by the presence of fewer sacules, thicker septa, and decreased width of the air space lumens. The animals in the nitrofen-EGFP group without hernias had

![Fig. 4. Histology of nitrofen-treated lungs following in utero cftr gene therapy. Animals were treated with nitrofen at 13 days gestational age (A–D) or carrier only (E and F). At day 16 of gestation animals were treated with either AdCMVEGFP (A, C, and E) or Av1CF2 (CFTR; B, D, and F). A, B: animals with CDH; C–F: animals without hernias. Hematoxylin and eosin stains; original magnification ×200.](image-url)
slightly improved histology (Fig. 4C) but continued to have fewer terminal bronchi and saccular dilatation that the controls. In contrast, the nitrofen animals treated with CFTR had thinner interstitium indicative of a more developed lung. Even if the nitrofen-CFTR animal had a CDH (Fig. 4B), there was improvement in histology with thinning of the interstitium and more saccular air space present. The histological appearance of nitrofen-CFTR animals (Fig. 4D) more closely matched the appearance of the control animals that had not been exposed to nitrofen (Fig. 4E).

**Volume proportion of tissues.** As can be expected by histology in Fig. 4, the volume proportion of saccular air was increased with CFTR treatment, and the volume proportion of parenchyma decreased with CFTR treatment. The thinning of the septa was indicative of more mature complex lung. This is demonstrated in Fig. 5.

**CFTR Gene Transfer**

In utero CFTR gene therapy at 16 days gestation resulted in marked improvement of lung development in the fetuses treated with nitrofen at 13 days gestation but negligible improvement following nitrofen treatment at 10 days gestation. CFTR mRNA was quantitated in the lungs of the fetuses to determine whether the treatment outcomes differed because of gene transfer efficiency. CFTR mRNA was measured in the lungs at 22 days gestation using real-time PCR in each of the groups. After nitrofen exposure at 10 days gestation, the relative expression of lung CFTR mRNA was not increased in the fetal group that received the CFTR gene compared with the group that received the EGFP control virus (0.2 ± 0.1, n = 8). However, after exposure to nitrofen at 13 days gestation, the relative expression of CFTR mRNA was increased (5.7 ± 1.8, n = 8) in the lungs of fetuses that received the CFTR virus over those that received the EGFP virus (P < 0.05). Therefore, in utero gene transfer efficiency to the lung was affected by the gestational age at which nitrofen exposure occurred. As disruption of lung development also correlates with the gestation at which nitrofen exposure occurs, these results confirmed previous findings that in utero gene transfer efficiency is affected by the developmental maturity of the lung (19).

**DISCUSSION**

Severe pulmonary hypoplasia remains a major cause of the high morbidity and mortality in CDH yet its precise etiology remains elusive (14). Treatment of lungs with in utero CFTR gene therapy at 16 days gestation following nitrofen treatment at 13 days resulted in marked improvement of fetal lung development. These changes were associated with high CFTR mRNA levels following gene transfer. The developmental changes were predominantly in the parenchyma or air-exchanging portion of the lung and would translate into improved infant survival. The gas-exchanging surface area and number of saccules per lung increased. In addition, the volume proportion of saccular airspace improved, rescuing the lungs from the compressed parenchyma that is seen in the nitrofen model (4).

Treatment of lungs with in utero CFTR gene transfer at 16 days gestation following nitrofen treatment at 10 days resulted in negligible improvement of fetal lung development and was not associated with changes in CFTR mRNA levels following gene transfer. The developmental changes were predominantly in the parenchyma or air-exchanging portion of the lung and would translate into improved infant survival. The gas-exchanging surface area and number of saccules per lung increased. In addition, the volume proportion of saccular airspace improved, rescuing the lungs from the compressed parenchyma that is seen in the nitrofen model (4).

Previous studies on in utero CFTR gene transfer were shown to result in a lethal phenotype in mice (7) due to cellular overgrowth. In rats, however, CFTR overexpression produced animals with increased resistance to *Pseudomonas* challenge. In this study, the CDH lung was shown to be developmentally immature and CFTR overexpression to overcome this defect. All these studies are consistent with CFTR’s acting as an accelerator of lung development.

The primary limitation of the nitrofen-induced rat hernia model is the involvement of multiple organ defects. Thus it is impossible to evaluate the long-term effects of in utero CFTR gene therapy on lung function. In addition, the gene therapy window in rats is much shorter than humans. As shown by the
gene transfer failure in 10-day nitrofen studies, hernia development can affect in utero delivery of the gene.

In the future, in utero gene therapy may provide a therapeutic option for infants with CDH, particularly because it can be performed within a therapeutic window following diagnosis. CFTR gene therapy could promote development of the lung to complement the postnatal surgical repair of the hernia. Recently, this laboratory performed experiments on CFTR-dependent gene expression in the fetal lung (Cohen and Larson, unpublished data). These data showed that CFTR is involved in regulation of stretch-induced differentiation of the lung by controlling airway smooth muscle contractions. Thus, in CDH, CFTR reversed the lung phenotype by augmenting stretch-induced differentiation. It is possible that in utero cftr gene therapy may be able to treat a spectrum of disrupted lung development syndromes.

GRANTS
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REFERENCES