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Prenatal treatment with retinoic acid promotes pulmonary alveologenesis in the nitrofen model of congenital diaphragmatic hernia☆

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Key words:
Congenital diaphragmatic hernia;
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Retinoic acid;
Nitrofen

Abstract

Background/Purpose: Severe pulmonary hypoplasia remains the main cause of the high mortality in newborn infants with congenital diaphragmatic hernia (CDH). Retinoids are a family of molecules derived from vitamin A, which play an important role in lung development. We hypothesized that retinoids promote alveologenesis at the end of gestation and therefore designed this study to investigate the effects of retinoid acid on nitrofen-induced hypoplastic lungs in CDH.

Methods: Pregnant rats were exposed to either olive oil or 100 mg nitrofen on day 9 of gestation. Retinoic acid 5 mg/kg was given intraperitoneally on days 18, 19, and 20 of gestation and fetuses were recovered on day 21. We had 4 study groups: control (n = 24), control + retinoic acid (n = 22), CDH (n = 24), and CDH + retinoic acid (n = 19). Lungs from the 4 study groups were fixed, and the following stereological measurements were performed on vertical random sections: total lung volume, volume density of airspaces, volume density of air walls, gas exchange surface area, alveolar volume, and total number of alveoli per lung. Total DNA content of each lung was measured using a spectrophotometer.

Results: Total lung volume increased in CDH lungs after the addition of retinoic acid but remained the same in the control group. Gas exchange surface area was larger in CDH lungs after the addition of retinoic acid but remained unchanged in the control group. The total number of alveoli per lung was higher after the addition of retinoic acid. Total DNA content as well as total DNA content–lung weight ratio of the left lung increased significantly in the CDH group after the addition of retinoic acid compared with CDH without retinoic acid.

Conclusions: Our results demonstrate that prenatal treatment with retinoic acid stimulates alveologenesis in hypoplastic lungs in CDH.

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Congenital diaphragmatic hernia (CDH) is a common congenital malformation with an incidence of 1 in 2500 newborns. Despite remarkable progress in resuscitation and intensive care, the morbidity and mortality rates in CDH remain high because of severe pulmonary hypoplasia [1]. Postnatal therapies such as extracorporeal membrane oxygenation, inhaled nitric oxide, surfactant, and high-frequency ventilation have had limited impact on prognosis, and survivors of CDH commonly face considerable morbidity [2,3]. Prenatal treatments have also been tried in CDH: open fetal diaphragmatic repair has not showed survival benefit [4], and a randomized controlled trial on fetoscopic tracheal occlusion has not shown to be beneficial [5]. Recently, a small-scale randomized controlled trial investigated the use of corticosteroids in late gestation in CDH and showed no benefit [6].

Retinoids are the family of molecules derived from vitamin A. Vitamin A is obtained from the diet in the form of retinyl esters present in animal meat or β-carotene present in vegetables. After absorption, retinyl esters are transported to the liver for storage, where they are metabolized into retinol [7]. Retinol bound to retinol-binding protein is transferred from the liver via blood to target cells. Within the cytoplasm, retinol is converted into retinal by retinol dehydrogenase and then into retinoic acid by retinal dehydrogenase. Retinoic acid is the biologically active metabolite of retinoids. It enters the nucleus and binds to 2 nuclear transcription factors known as retinoic acid receptors (RARs) and retinoid X receptors (RXRs). There are 3 members of each class of receptor and these are known as α, β and γ, thus giving RARα, RARβ, RARγ and RXRα, RXRβ, RXRγ. The RARs and RXRs are the functional units in transducing the retinoid signal at the gene level [7].

Retinoids are essential for normal development of various organs including the lungs and diaphragm during embryogenesis [8]. In lung development, retinoids play an important role in each of the lung developmental stages. The agenesis of the lung buds seen in embryos under conditions of acute maternal retinol deficiency highlights the importance of retinoids in the stimulation of the initial budding of the lungs [9]. A major role for retinoic acid in early lung morphogenesis is to selectively maintain mesodermal proliferation and induce fibroblast growth factor 10 expression in the foregut region in the initial budding of the lungs from the foregut [10]. During the subsequent pseudoglandular period, dichotomous branching forms the conducting airways. This process is highly dependent on epithelial-mesenchymal interactions and retinoids play a crucial role [11]. In the canalicular period, the airways become larger and the vascularity increases. During this period, RARβ, and RXRα and RXRβ are associated with epithelial cell differentiation and structural changes [9]. The saccular period is characterized by a further subdivision of bronchioles and the formation of primitive alveoli. During this period, retinoic acid stimulates proliferation of type II cells through an epithelial growth factor–mediated pathway [12]. During the alveolar period, there is a decrease of retinyl ester storage and production of the active metabolite, retinoic acid [9]. Strong evidence for a role of retinoids in alveologenesis comes from a study from Massaro and Massaro [13]. These authors demonstrated a 50% increase in alveolar number along with a reduction in their size if retinoic acid was given to newborn mice. Moreover, recent data suggest that retinoic acid can induce alveolar regeneration in the adult mouse lung [14].

In the 1940s, dietary deprivation studies first demonstrated that diaphragm development was linked to retinoids. Diaphragmatic hernias were present in 25% to 75% of the offspring of vitamin A–deficient dams [15,16]. Studies on RARs double knockout mice have demonstrated that some offspring have CDH. Interestingly, RAR $\alpha\beta$2 double mutant had right-sided defects, whereas RAR $\alpha\beta$2+/− double mutant had left-sided defects [17]. A small clinical study showed that infants with CDH had 50% less plasma retinol and retinol-binding protein levels compared with levels in healthy infants [18].

Nitrofen (2,4-dichlorophenyl-p-nitropheniyl ether) is an herbicide that has been used for many years to create an animal model of CDH [19]. Nitrofen has a low toxicity in adult rodents, but its administration to pregnant rodents in a specific period during gestation results in a high rate of CDH and associated pulmonary hypoplasia to their embryos that is strikingly similar to the human malformation [19]. It has been demonstrated that the incidence of nitrofen-induced CDH in rats can be reduced from 80% to 50% if large doses of vitamin A are administered antenatally [20]. Moreover, the incidence of CDH can be dramatically reduced to 8% if retinoic acid is given to pregnant rats along with nitrofen [21]. Collectively, these data strongly imply that retinoids are candidates for involvement in the pathogenesis of CDH in the nitrofen model. Recently, Nakazawa et al [22] have demonstrated that lung retinol storage is decreased and gene expression of most downstream components of the retinoid signaling pathway is increased in the nitrofen model of CDH. These results strongly suggest that nitrofen acts by interfering with the cellular uptake of retinol resulting in CDH in this model.

In clinical practice, the prenatal diagnosis of CDH occurs when the actual diaphragmatic hernia is already produced. Because most newborns with CDH die primarily of respiratory failure secondary to severe pulmonary hypoplasia, the therapeutic approach should be focused on reverting lung hypoplasia and promoting lung growth at the end of gestation.

We hypothesized that retinoic acid, the active metabolite of retinoids, can revert lung hypoplasia and enhance lung growth if given during late gestation in the nitrofen model of CDH. Therefore, we designed this study focusing on signs of cell proliferation and alveologenesis using DNA content measurement and stereological tools.

1. Materials and methods

1.1. Animals and drugs

Adults Sprague-Dawley rats were mated overnight. Twelve hours later, the presence of spermatozoids in the vaginal smear was verified and was considered as gestational day 0. Pregnant female rats were then randomly divided into 2 groups. Animals in the experimental group received intragastrically 100 mg of nitrofen (Wako Chemicals, Osaka, Japan) dissolved in 1 mL of olive oil on day 9.5 of gestation, whereas those in the control group received only vehicle. On gestational day 18, the rats were randomly injected intraperitoneally with all *trans*-retinoic acid 5 mg/kg in cottonseed oil (Sigma, St Louis, Mo) or with diluent according to previously described protocols [23]. The injections were repeated on days 19 and 20. At term (day 21), the rats were sedated with isofluorane and then killed by intracardiac injection of sodium pentobarbital. The fetuses were then recovered by cesarean delivery, and the diaphragm was carefully examined for the presence of a hernia under a dissecting microscope (Leica S8 APO, Heerbrugg, Switzerland). We analyzed the left lungs of both control and CDH groups. This provided 4 treatment groups: control (n = 24), control + retinoic acid (n = 22), CDH (n = 24), and CDH + retinoic acid (n = 19). To obtain representative study groups, the fetuses in each group came from at least 8 different dams. Lungs were processed for stereological and DNA measurements. The Department of Health and Children approved all the animal experiments (reference no. B100/3530) under the Cruelty to Animals Act, 1876; as amended by European Communities Regulations 2002.

1.2. Fixation and tissue sampling for stereology

After assessing the presence of CDH, the thorax was opened through an anterior midline incision and the trachea was dissected at the level of the neck, where it was transversally cut. Buffered formalin was infused into the trachea at a pressure of 20 cm H_2O. The trachea was then ligated, the lungs were removed from the thorax, and fixation was continued for 2 hours. After fixation, the left lungs in all groups were dissected and freed from the mediastinal structures. To obtain vertical uniform random sections, the lung was rotated about a vertical axis and then embedded in paraffin.

1.3. Stereological measurements

The stereological measurements were performed by one observer (SM) who was blinded to which group the samples belonged. Total lung volume was calculated with the Cavalieri method [24] as follows. Each lung was exhaustively sectioned at 5 μm. A random number between 0 and 39 was used to determine the first section sampled for counting to ensure a random position of the sections within the lung. Every 40th section thereafter was sampled. This yielded between 9 and 14 sections per lung. A point graticule (1 mm distance) was placed on top of each slide, and the number of points hitting the section was then counted. The volume of each lung was calculated using the formula: $V_L = T \cdot a/p \cdot \Sigma P$, where V_L is lung volume; T is the distance between sections, in this case 200 μm (40 sections of 5 μm each); a/p is the area associated with each point, in this case 1 mm^2; and ΣP is the sum of all points hitting the sections.

For the subsequent stereological measurements, 3 random points were placed in each of the lung sections using Stereology Pro 5.1 software incorporated in Image Pro Plus 5.0 (Media Cybernetics, Silver Spring, Md) and were photographed at 200 times magnification preserving the verticality of the sections. Therefore, we analyzed between 27 and 42 images per lung.

Volume density (Vv) was determined using a point graticule placed on top of each photograph by counting the number of points that fell on airspaces, airspace walls, bronchi, and vessels divided by the total number of test points [24].

Surface density was determined using a cycloid graticule with an associated point grid using the formula: $S = (2 \cdot \Sigma I)/(l/p \cdot \Sigma P)$, where S is the total surface density of the lung, ΣI is the sum of the number of intersections between the airspace surface and the cycloid probe, l/p is the length of the test line per grid point corrected for linear magnification, and ΣP is the is the sum of all points hitting the reference space. Total gas exchange area was calculated by multiplying surface density by total lung volume [24].

Mean alveolar volume was determined using the point sampled intercept method. All the points hitting airspaces were selected using a point grid. A straight line was then drawn through each point intersecting 2 opposite ends of the airspace wall. Two other lines, both passing through the selected point and orientated 60° from each other, were drawn. The length of these 3 lines was measured and an average length was calculated. Mean alveolar volume was determined using the formula: $V_{al} = (\pi/3 \cdot n) \cdot \Sigma l^3$, where V_{al} is mean alveolar volume, "n" is the total number of the point sampled linear intercepts, and Σl^3 is the sum of the cubed point sampled intercept length already averaged [24].

The total number of alveoli per lung was calculated using the formula:

$$N = (V_L \cdot \text{Vv airspaces})/V_{al},$$

where V_L is total lung volume, Vv airspaces is the volume density of the airspaces, and V_{al} is the mean alveolar volume [13,24].

1.4. Fixation and tissue sampling for DNA measurement

After assessing the presence of CDH, lungs were weighted, transferred into a 1.3-mL microtube, and stored at

−80°C for further use. Total DNA of each lung was extracted using a commercially available kit (QIAGEN Genomic-tip 100/G and Genomic DNA Buffer Set, QIAGEN, Crawley, UK). Total DNA content was measured using a spectrophotometer (Nano Drop ND-1000, Wilmington, Del).

1.5. Statistical methods

For each parameter measured or calculated from measurements, the mean and SD were calculated. Normality of the groups was checked using Kolmogorov-Smirnov test. Differences between the groups were tested by 1-way analysis of variance. Statistical significance was defined as $P < .05$. Data were analyzed using Statistical Package for the Social Sciences 11.0 for Windows program (SPSS, Chicago, Ill).

2. Results

2.1. Lung volumes

Total lung volume was significantly reduced in CDH lungs compared to control lungs (8.32 ± 0.78 mm^3 [CDH] vs 19.56 ± 3.01 mm^3 [control], $P < .001$). Total lung volume did not differ after the addition of retinoic acid in control group (19.56 ± 3.01 mm^3 [control] vs 17.04 ± 1.43 mm^3 [control + retinoic acid], $P = .2$). Total lung volume significantly increased after the addition of retinoic acid in CDH group (8.32 ± 0.78 mm^3 [CDH] vs 12.93 ± 2.55 mm^3 [CDH + retinoic acid], $P < .05$). However, total lung volume of CDH after the addition of retinoic acid did not reach the control lung volume (12.93 ± 2.55 mm^3 [CDH + retinoic acid] vs 19.56 ± 3.01 mm^3 [control], $P = .0008$). Volume density of airspaces was significantly reduced by 21% in both control and CDH groups after the addition of retinoic acid (Table 1). Conversely, retinoic acid treatment significantly increased the volume density of the air walls by 23% in control group and by 14% in CDH groups. Volume density of bronchi and vessels did not vary (Table 1 and Fig. 1).

2.2. Gas exchange surface area

Total gas exchange surface area did not vary in the control group after the addition of retinoic acid. However, it increased by 85% in CDH group after retinoic acid treatment, reaching the values of the control lungs (Fig. 2).

2.3. Airspaces size and number

Airspaces in CDH lungs were smaller than airspaces in control lungs. After retinoic acid treatment, mean airspace volume significantly decreased in both control and CDH groups (Fig. 3). The administration of retinoic acid resulted in a significantly higher absolute number of airspaces in both control and CDH groups (Fig. 4).

2.4. DNA measurements

Total DNA significantly increased in both groups after the addition of retinoic acid. However, total DNA content–lung weight ratio significantly increased only in the CDH group after the addition of retinoic acid (Table 2).

3. Discussion

Alveolization starts with the appearance of alveolar septa, which divide the primitive air sac of the saccular phase of lung development into smaller units, the alveoli. These new walls are called *secondary septa*. The alveolar surface area therefore increases substantially as the diameter of the airspaces decreases [25]. The final remodeling of the alveolar walls to assume the adult characteristic morphology represents the last stage in lung development. The cells that make up the walls of the developing alveoli show interesting and important features. The type I cell is the fully differentiated squamous epithelial cell with thin attenuated cytoplasm forming the barrier between the air and the capillary. There is little metabolic activity in this cell, whose function is to provide a surface of minimal thickness between

Table 1 Volume density of lung components

	Volume density of airspaces	Volume density of air walls	Volume density of bronchi	Volume density of vessels
Control (n = 7)	0.5041 ± 0.0605	0.3835 ± 0.0684	0.0745 ± 0.0231	0.0398 ± 0.0328
Control + retinoic acid (n = 7)	0.3966 ± 0.0224 *, †	0.5004 ± 0.0051 **, †	0.0893 ± 0.0189	0.0211 ± 0.0089
CDH (n = 7)	0.441 ± 0.0689	0.4647 ± 0.0717	0.0709 ± 0.0099	0.0245 ± 0.0159
CDH + retinoic acid (n = 7)	0.3435 ± 0.0355 ***, ‡‡	0.5424 ± 0.0362 ***, ‡	0.0815 ± 0.0125	0.0216 ± 0.0106

* $P < .05$ vs control group.
** $P < .01$ vs control group.
*** $P < .001$ vs control group.
† $P < .01$ vs CDH + retinoic acid group.
‡ $P < .05$ vs CDH group.
‡‡ $P < .01$ vs CDH group.

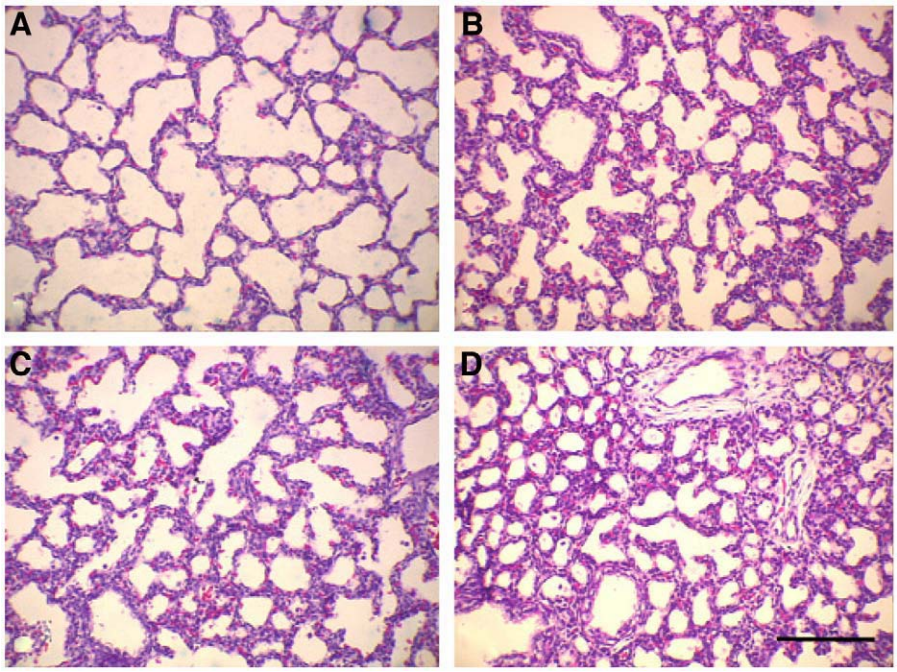

Fig. 1 Histologic sections of lung. A, Control lung in a term rat fetus. B, Control lung with added retinoic acid. C, Congenital diaphragmatic hernia lung in a term rat fetus. D, Congenital diaphragmatic hernia lung with added retinoic acid. Note that airspaces look smaller and the airspace walls look thicker in B and D compared to A and C, respectively (hematoxylin-eosin staining; scale bar, 100 μm).

the alveolar gas and the pulmonary capillaries [25]. The type II cell produce pulmonary surfactant and is regarded to be the progenitor or stem cell for the type I cell [26]. Type II cells accounts for approximately 50% of the alveolar epithelial cell numbers but only 7% of the surface area. These cells are near connective tissue cells lying beneath them. There are 2 types of connective tissue cells. In one, there are abundant lipid droplets, the lipid-laden interstitial fibroblasts, which store retinoids and are often in close contact with type II cells [27,28]. The other population of cells is the non–lipid-laden interstitial fibroblasts. Together, these cells are the major contributors to the synthesis of extracellular matrix, structural proteins such as collagen and elastin, which provide tensile strength and elasticity to the gas-exchanging surface [29,30].

Based on the recent evidence involving retinoids in the pathogenesis of CDH [20-22], some investigators have studied the effects of retinoids in late gestation in the nitrofen model of CDH. Baptista et al [31] failed to demonstrate any changes in total lung-to-body weight ratio at term when vitamin A was given to nitrofen-treated pregnant rats on gestational day 18. Gonzalez-Reyes et al [32] did not find any changes in lung weight-to-body weight ratio either, but they demonstrated an increase in total DNA and a normalization in thyroid transcription factor 1, hepatocyte nuclear

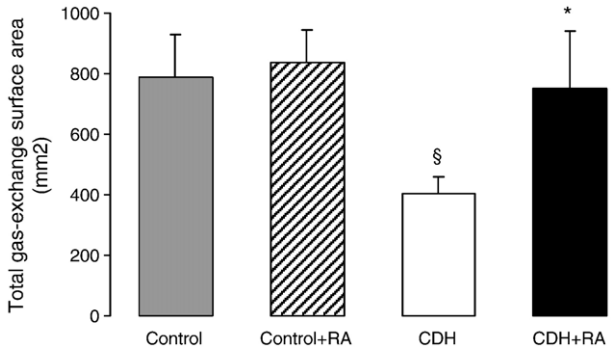

Fig. 2 Total gas exchange surface area in lungs. Total gas exchange surface area did not vary in control group after the addition of retinoic acid. Gas exchange surface area significantly increased in CDH group after the addition of retinoic acid reaching the value of control lungs. $^{\S}P < .01$ vs control and control + RA; $^{*}P < .05$ vs CDH group. RA indicates retinoic acid.

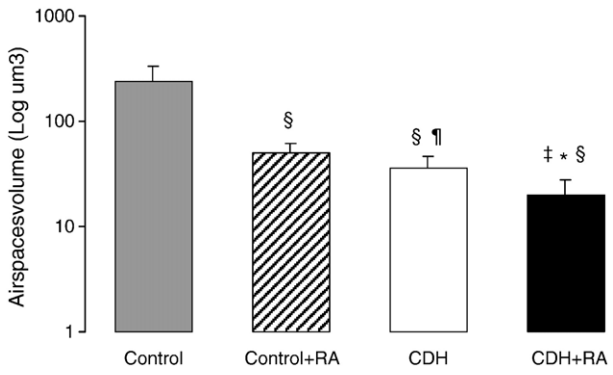

Fig. 3 Lung airspace volume (logarithmic transformation). The addition of retinoic acid significantly decreased the airspace volume in both groups. $^{\S}P < .01$ vs control group; $^{\P}P < .05$ vs control + RA group; $^{*}P < .05$ vs CDH group; $^{\ddagger}P < .01$ vs control + RA group.

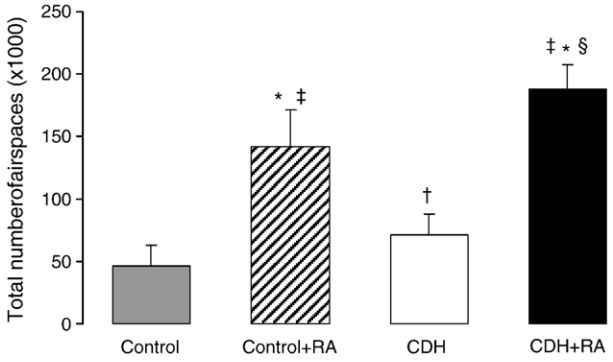

Fig. 4 Total number of airspaces in lungs. Total number of airspaces significantly increased after the addition of retinoic acid in both groups. *$P < .01$ vs control group; ‡$P < .01$ vs CDH group; †$P < .05$ vs control group; §$P < .05$ vs control + RA group.

factor 3β, and surfactant protein B after treatment with vitamin A on days 16 to 18 of gestation.

Retinoic acid is the active metabolite of the retinoid pathway. It has been demonstrated that the lipid-laden interstitial fibroblasts synthesize and secrete retinoic acid [33], which induces proliferation of type II cells and enhances tropoelastin gene expression in vitro [12,34]. Retinoic acid has also been demonstrated to be a powerful promotor of alveologenesis in newborn rats and also in the adult mouse lung [13,14]. These reasons led us to consider that treatment with retinoic acid, rather than with vitamin A, may promote alveologenesis in the nitrofen model of CDH.

In fetal rat lungs, the concentration of retinyl palmitate and total retinyl ester levels increases rapidly to a peak on gestational day 18. There is a steady decline thereafter reaching a nadir after birth [35-37]. Based on these findings, we chose embryonic days 18, 19, and 20 of gestation for retinoic acid treatment.

Stereology is a morphometric technique that uses geometric probes for sampling structures and has dramatically improved over the last 15 years [24]. This technique allows unbiased and representative estimates of nearly all morphometric parameters such as volume, surface, length, and number of structures. Particularly in the lung, stereology has increased the rigor and accuracy of the study of the formation of alveoli [38]. Using this technique, we were able to perform measurements on lung volumes, gas exchange surface area, and airspace size and number.

By measuring volume density, we quantified the proportional amount of the different types of structures in the lung: airspaces, airspace walls, bronchi, and vessels. We found an inverse relationship between volume density of airspaces and volume density of airspace walls after treatment with retinoic acid. Airspace walls and septi occupied more space in the lung after retinoic acid addition, whereas airspaces occupied less space in both control and CDH lungs. This findings clearly reflect to what other authors have previously showed regarding the effects of retinoic acid as promotor of secondary septation and alveolus formation in the lung [13,29]. The increased DNA concentration in the lungs after retinoic acid treatment reflects an increased cell number. Because volume density of bronchi and vessels did not change, this increased cell number is most likely secondary to the enlarged airspace septi and walls. Other authors have recently found an increased total DNA after vitamin A treatment in the nitrofen model of CDH [32]. As a consequence of the secondary septation, airspaces were smaller and more numerous after the addition of retinoic acid as reflected in airspace volume and total number of airspace measurements, respectively. Surprisingly, there were a higher number of alveoli in CDH lungs compared to control lungs. However, we believe this is because total number of airspaces is the result of an indirect measurement influenced directly by total lung volume and volume density of airspaces and inversely by mean alveolar volume. Because airspaces in CDH lungs are hypoplastic compared to controls, mean alveolar volume is reduced, and, therefore, it affects the calculation on total number of alveoli per lung. Probably, a direct measurement of number of alveoli with stereological tools would solve this issue.

In species with little locomotive activity at birth, notably the mouse and the rat, true alveoli with thin walls are not present at birth. Alveolar stage of lung development takes place postnatally from day 4 to 14 [39]. In our model we harvested the fetuses at term, and, therefore, the lungs did not complete alveologenesis. We believe this is the reason why although retinoic acid promoted cell proliferation and secondary septation in our study, septi were thicker and, therefore, the histologic appearance of the lung was somehow "immature." However, we used rats in our study because along with mice, these are the only animal model available so far for studying lung development in CDH.

Total gas exchange surface area significantly increased in CDH lungs after retinoic acid treatment. Interestingly, this parameter remained unchanged in control lungs. Massaro and Massaro did not find an increased gas exchange surface area either after treatment with retinoic acid in control newborn rats [13]. At that time, they proposed that their result could reflect the presence of a control mechanism that

Table 2 Total DNA in lungs

	Total DNA content(μg)	Total DNA/lung weight
Control (n = 17)	86.73 ± 23.44	2.041 ± 0.768
Control + retinoic acid (n = 15)	111.44 ± 19.16 **	2.188 ± 0.359
CDH (n = 17)	67.44 ± 16.24 *, †	3.306 ± 0.567
CDH + retinoic acid (n = 12)	96.19 ± 13.70 ‡‡	3.951 ± 0.464 ‡

* $P < .05$ vs control group.
** $P < .01$ vs control group.
† $P < .01$ vs control + retinoic acid group.
‡ $P < .05$ vs CDH group.
‡‡ $P < .01$ vs CDH group.

inhibits the size of alveoli when there is no need for additional gas exchange surface area. After that they demonstrated that in RARβ −/− mice, alveolar volume was smaller, there were more alveoli, and the alveolar surface area was larger, suggesting that RARβ is the endogenous inhibitor of alveolar septation [40]. Because lungs are hypoplastic in CDH, this physiologic regulatory mechanism might be altered, and, therefore, there are no restrictions for an increased gas exchange surface area.

Corticosteroids have been widely used in preterm newborns with bronchopulmonary dysplasia to accelerate maturation and surfactant synthesis and to prevent inflammatory processes [41]. In the animal model of CDH, corticosteroids have been used to try promoting lung growth in uterus [42,43]. However, a recent clinical trial on the prenatal use of corticosteroids in human CDH failed to demonstrate any benefits [6]. There is increasing evidence to suggest that corticosteroids actually promote maturation of lung by accelerating alveolar wall thinning and fusion of the 2 capillary layers but at the same time by inhibiting outgrowth of new septa leading to early termination of the alveologenesis process [41,44-46]. Importantly, retinoic acid treatment antagonizes corticosteroids effects and partially rescues failed septation induced by corticosteroids in mice and rats [13,47]. The underlying mechanisms of these antagonistic effects are not completely understood, but it is likely that there is a balance between retinoic acid and corticosteroids in the course of normal lung development [41].

In the clinical setting, blood retinol concentration has been recognized to be lower in preterm newborns than in full-term infants and in those who develop bronchopulmonary dysplasia than in those who do not [48,49]. In trials of vitamin A supplementation, reduced need for supplemental oxygen and mechanical ventilation was observed, but whether incidence of bronchopulmonary dysplasia was reduced remains controversial with either no change or slight decrease [50-52]. With regard to CDH, there is only one clinical study that demonstrated there was a 50% reduction in plasma retinol and retinal-binding protein levels in a group of 11 CDH newborn compared to matching controls [18]. These clinical data along with the extensive experimental evidence in the animal model of CDH strongly support the idea of a retinoid signaling pathway impairment in CDH. Our results demonstrate that prenatal treatment with retinoic acid in late gestation can revert lung hypoplasia and enhance lung growth in the nitrofen rodent model of CDH. These findings provide the first experimental support for the possibility that a pharmacologic agent may provide remedial therapy in late gestation in human CDH.

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