

## *Pre- and Postnatal Lung Development, Maturation, and Plasticity* Effects of oligohydramnios on lung growth and maturation in the fetal rat

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**Kitterman, Joseph A., Cheryl J. Chapin, Jeff N. Vanderbilt, Nicolas F. M. Porta, Louis M. Scavo, Leland G. Dobbs, Robert Ertsey, and Jon Goerke.** Effects of oligohydramnios on lung growth and maturation in the fetal rat. *Am J Physiol Lung Cell Mol Physiol* 282: L431–L439, 2002. First published October 5, 2001; 10.1152/ajplung.00161.2001.—Oligohydramnios (OH) retards fetal lung growth by producing less lung distension than normal. To examine effects of decreased distension on fetal lung development, we produced OH in rats by puncture of uterus and fetal membranes at 16 days of gestation; fetuses were delivered at 21 or 22 days of gestation. Controls were position-matched littermates in the opposite uterine horn. OH lungs had lower weights and less DNA, protein, and water, but no differences in saturated phosphatidylcholine, surfactant proteins (SP)-A and -B, and mRNA for SP-A, -B, -C, and -D. To evaluate effects on epithelial differentiation, we used RTI<sub>40</sub> and RTII<sub>70</sub>, proteins specific in lung to luminal surfaces of alveolar type I and II cells, respectively. At 22 days of gestation, OH lungs had less RTI<sub>40</sub> mRNA ( $P < 0.05$ ) and protein ( $P < 0.001$ ), but RTII<sub>70</sub> did not differ from controls. With OH, type I cells (in proportion to type II cells) covered less distal air space perimeter ( $P < 0.01$ ). We conclude that OH, which retards lung growth, has little effect on surfactant and impedes formation of type I cells relative to type II cells.

fetal lung development; lung distension; pulmonary epithelial differentiation; pulmonary hypoplasia; pulmonary surfactant

IN LATE GESTATION, the fetal lung undergoes marked changes in preparation for the transition to extrauterine life. These changes include growth, enlargement of distal potential air spaces, thinning of the septa, maturation of the surfactant system, and differentiation of

distal pulmonary epithelium into mature alveolar type I and type II cells. Several studies have shown that fetal lung growth is controlled primarily by mechanical factors, especially distension of the lung (for reviews, see Refs. 26 and 29) and that maturation of the surfactant system is controlled primarily by endocrine factors (for reviews, see Refs. 3 and 48). In contrast, relatively little is known about factors that regulate differentiation of the alveolar epithelium.

In 1977, Alcorn and associates (2) reported that tracheal ligation in fetal sheep, which increased lung distension, caused accelerated lung growth and a qualitative reduction in the number of alveolar type II cells; conversely, chronic drainage of tracheal fluid, which inhibited lung distension, retarded lung growth and increased the number of type II cells. However, they reported neither quantitative data for cell counts nor measurements of indicators of surfactant, which is produced by type II cells. Subsequently, other investigators have studied tracheal ligation in fetal sheep and have confirmed that an increase in lung distension results in a lower number of type II cells (6, 11, 37) and a higher percentage of type I cells (18). Tracheal ligation also results in lower concentrations in the lung of surfactant protein (SP)-A and saturated phosphatidylcholine (SatPC), the major surface-active lipid in pulmonary surfactant (28), as well as mRNA for SP-A, -B, and -C (32). Conversely, transection of the cervical spinal cord (which abolishes fetal breathing movements) produces hypoplastic lungs with higher concentrations of SP-A and SatPC (28).

Oligohydramnios, a deficiency of amniotic fluid, when prolonged, results in pulmonary hypoplasia in

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human fetuses (19, 39). Experimentally induced oligohydramnios retards fetal lung growth in guinea pigs (36), monkeys (25), rabbits (1), rats (35) and sheep (12). The likely mechanism by which oligohydramnios retards lung growth is by reducing the volume of fluid in the potential airways and air spaces (12, 40), thereby reducing distension of the fetal lung.

On the basis of the studies cited above, we hypothesized that a lower degree of distension of the fetal lung due to oligohydramnios would not only retard lung growth but would also cause a relative increase of type II cells in the distal pulmonary epithelium and accelerate formation of pulmonary surfactant. To test these hypotheses, we have studied the effects of oligohydramnios on the lung in fetal rats.

## METHODS

All studies were approved by the Committee on Animal Research of the University of California, San Francisco, and all procedures conformed with the *NIH Guide for the Care and Use of Laboratory Animals*. Timed-pregnant Sprague-Dawley rats were obtained from Charles River Laboratories (Hollister, CA) on *day 14* of gestation and were provided feed and water ad libitum. Ambient temperature was maintained at 21°C.

We studied 204 fetuses from 56 pregnant dams. On *day 16* of gestation, dams and fetuses were anesthetized with a maternal intramuscular injection of ketamine (Parke-Davis, Morris Plains, NJ; 90 mg/kg) and xylazine (Butler, Columbus, OH; 1.25 mg/kg). Oligohydramnios was produced as previously described (7, 35, 46) with minor modifications. Briefly, through a midline laparotomy, the right or left uterine horn, alternately, was exposed, and the uterine wall and fetal membranes of each uterine sac in one horn were punctured using a sterile 22-gauge needle. This procedure resulted in immediate visible leakage of amniotic fluid and a decrease in size of the uterine sac. Fetuses in corresponding positions in the opposite uterine horn served as controls. We counted and recorded the number of fetuses in each horn. The uterus was then returned to the abdomen, the abdominal incision was repaired in two layers with silk sutures, and the dam was allowed to recover.

We delivered litters of fetuses on either *day 21* or *day 22*. Before delivery, dams and fetuses were again anesthetized by intramuscular injection of ketamine (180 mg/kg) and xylazine (2.5 mg/kg). The fetuses were removed by hysterotomy and were managed either for lung biochemical or morphological studies or for collection of blood for measurement of corticosterone. For biochemical studies of the lungs, fetuses were weighed and pithed, and their lungs were removed, weighed, and snap-frozen in liquid nitrogen. Techniques for the other studies are described below. After all fetuses had been removed, dams were killed by intracardiac injection of pentobarbital sodium (Euthanasia injection, Anthony Products, Arcadia, CA; 1 ml, 390 mg/ml solution) followed by production of bilateral pneumothoraxes.

**Biochemical studies.** To estimate dry lung weight, previously weighed fetal lungs were dried to constant weight in a vacuum oven at 86°C. To quantify DNA, frozen lungs were homogenized by ultrasonication, and DNA content was measured using the fluorometric method of Setaro and Morley (43). Protein in lung tissue was measured with a commercially available kit (Sigma, St. Louis, MO) using the bicinchoninic acid (BCA) modification of the Lowry method (44).

**Corticosterone measurements.** To determine if oligohydramnios affected fetal glucocorticoids, which could in turn affect lung maturation, corticosterone was measured in serum from oligohydramnios fetuses and control fetuses delivered at either *day 21* or *day 22*. After delivery, the fetuses were immediately decapitated with sharp scissors, and blood was collected by drainage from the body. Serum was separated and frozen. Corticosterone was measured by radioimmunoassay using a specific antiserum for corticosterone by Endocrine Sciences (Calabasas Hills, CA).

**SatPC determination.** Lipids were extracted from whole fetal lung by the method of Bligh and Dyer (8) with the modification of addition of KCl granules to assist in separation of the two phases. The lipids were filtered (Whatman no. 1 filter paper), dried under nitrogen, and dissolved in a known volume of chloroform-methanol (20:1 vol/vol) to determine total lipid weight on a microbalance. To calculate phospholipid content, we measured phosphorus in an aliquot (5). SatPC was measured using the osmium tetroxide method (34) as previously described for fetal sheep lungs (30). Results are expressed as milligrams per gram of dry lung weight.

To evaluate the effects of oligohydramnios on the distal pulmonary epithelium, we used methods described below to measure RTI<sub>40</sub> and RTII<sub>70</sub>, proteins specific in lung to the apical membranes of rat type I and type II pneumocytes, respectively (13, 20), and mRNA for RTI<sub>40</sub> (47).

**Isolation and analysis of RNA.** RNA was isolated from lung by the RNazol (Tel-Test, Friendswood, TX) method, with the modification that 40 U of RNasin (Promega, Madison, WI) RNase inhibitor was added directly to the pellet before resuspension in diethyl pyrocarbonate-treated water containing 1 mM dithiothreitol. RNA was quantified by spectrophotometry [optical density at 260 nm (OD<sub>260</sub>)], and purity was ascertained using the OD<sub>260/280</sub> ratio. Values ranged from 1.7 to 2.0, with 1.9 being considered free of DNA and protein contaminants. RNA integrity was assessed by electrophoresis through formaldehyde agarose gels followed by ethidium bromide staining to visualize rRNA bands.

RNase protection assay probes for rat SP-A (16) and SP-B (15) were kind gifts of Dr. J. Shannon (University of Cincinnati, Cincinnati, OH). Rat SP-C probe was constructed by cloning a *Bam*HI fragment of the rat SP-C cDNA (17), corresponding to nt 505–748, into pBluescript SK(–) (Stratagene, La Jolla, CA). 18S rRNA probe template, pTRI RNA 18S, was purchased from Ambion (Austin, TX). <sup>32</sup>P-labeled antisense riboprobes were generated from linearized plasmids using MAXIscript in vitro transcription kit (Ambion). The resulting antisense transcripts for rat SP-A, SP-B, and SP-C mRNA and 18S rRNA were 146, 176, 300, and 158 nt, respectively. Gel-purified riboprobes were hybridized to total lung RNA (0.1, 0.05, and 0.025 µg) overnight at 42°C, digested with RNase A/T1, and separated on a 5% (SP-C with 18S) or 7.5% (SP-A and SP-B with 18S) polyacrylamide gel containing 8 M urea. Resulting protected fragments for rat SP-A, SP-B, and SP-C mRNA and 18S rRNA (139, 169, 238, and 80 nt, respectively) were visualized by autoradiography and quantified using a phosphorimager and Imagequant software (Molecular Dynamics, Sunnyvale, CA). Yeast tRNA was used in the hybridization reaction as a negative control. To correct for load of total RNA, surfactant protein mRNA levels were normalized to 18S rRNA.

RT-PCR was used for semiquantitative analysis of rat SP-D and RTI<sub>40</sub> mRNA content in fetal lung as previously described (41). Rat SP-D primers corresponding to nt 707–728 (sense 5'-AACTTCAGCGTCTAGAGGCTGC-3') and nt 1071–1092 (antisense 5'-ACAAGCCTTGTCATTCCACTGC-

3') and RTI<sub>40</sub> primers corresponding to nt 210–229 (sense 5'-CTAGCTGCTGAGGCTCCAAC-3') and nt 733–754 (antisense 5'-GGCGAGAACCTTCCAGAAATC-3') (47) were used to amplify total lung RNA. A linear range was established for each gene product by varying total RNA per reaction (0.125 and 0.250 µg) and/or cycle number (12, 15, 18, 21, and 25). The optimal conditions for SP-D were determined to be 0.125 µg total lung RNA through 15 cycles of PCR. Optimal conditions for RTI<sub>40</sub> were 0.250 µg total lung RNA followed by 15 cycles of PCR. RT-PCR products were subjected to electrophoresis through a 2% agarose gel, Southern blotted, probed, and washed as previously described (41). The oligonucleotides used as probes were unique for an internal sequence in each amplified product with rat SP-D probe corresponding to nt 821–840 (5'-AGGATGCCAAGGAGATGTGC-3') and RTI<sub>40</sub> probe corresponding to nt 485–504 [5'-GAACTG(CT)C-(TGC)ACCTCAG(TGC)AAC-3']. Blots were visualized by autoradiography and quantified by phosphorimager analysis as above.

**Quantification of proteins RTI<sub>40</sub>, RTII<sub>70</sub>, SP-A, and SP-B.** Whole fetal lung samples were Dounce homogenized in 1 ml 50 mM NaHCO<sub>3</sub>, pH 9.0, containing 1 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide, and 1 mM benzamidine HCl (Sigma). Homogenates were microcentrifuged for 30 s at 16,000 g, and the supernatant was assayed for protein concentration by the BCA method as above. Equal amounts of protein (8, 4, 2, and 1 µg) diluted in 1% MEGA-8 (Calbiochem-Novabiochem, La Jolla, CA)-4.5 M urea-25 mM NaHCO<sub>3</sub>, pH 9.0, were loaded in duplicate onto Nitrocellulose-1 membranes using the HYBRI-DOT 96-well filtration manifold (GIBCO BRL, Grand Island, NY). Membranes were incubated in 15% H<sub>2</sub>O<sub>2</sub> for 20 min at room temperature to block endogenous peroxidase activity, washed with 20 mM Tris-buffered 0.9% NaCl, pH 7.4 (TBS), and blocked with 5% nonfat dry milk-0.4% fish gelatin (Amersham Pharmacia Biotech, Piscataway, NJ)-0.3% BSA/TBS for 60 min at room temperature or overnight at 4°C. Blots were then washed once in TBS and incubated in primary antibody for 90 min at room temperature using the following dilutions: anti-RTI<sub>40</sub> 1:3,000, anti-RTII<sub>70</sub> 1:3,000, anti-SP-A 1:2,000, and anti-SP-B 1:5,000 in TBS. Monoclonal antibodies against RTI<sub>40</sub> and RTII<sub>70</sub> were produced in our laboratory as previously outlined (13, 20). Rabbit polyclonal antibodies against sheep SP-A and sheep SP-B (28) are immunoreactive to rat SP-A and rat SP-B by immunohistochemistry and Western blot analysis and were kind gifts of Dr. S. Hawgood (Univ. of California, San Francisco). Blots were washed six times over a 60-min period with TBS containing 0.05% Tween 20 (TBS-T), incubated in horseradish peroxidase-linked secondary antibody diluted 1:3,000 in TBS-T, and washed extensively with TBS-T. We used the following secondary antibodies: RTI<sub>40</sub>, sheep anti-mouse IgG (Amersham Pharmacia Biotech); RTII<sub>70</sub>, goat anti-mouse IgG<sub>3</sub> (Boehringer Mannheim, Indianapolis, IN); and SP-A and SP-B, donkey anti-rabbit IgG (Amersham Pharmacia Biotech). Blots were then developed with ECL(+) (Amersham Pharmacia Biotech), visualized by autoradiography, and quantified using a phosphorimager equipped with a blue fluorescence/chemifluorescence detector and Imagequant software (Molecular Dynamics).

**Morphological studies.** For morphological studies, we studied 10 fetuses: 5 fetuses exposed to oligohydramnios from 16 days of gestation and their controls. Fetuses were delivered at 22 days. After the fetus had been pithed, the trachea was cannulated through the larynx with a 24-gauge teflon catheter (Angiocath, Becton-Dickinson, Franklin Lakes, NJ), and the lungs were fixed in situ at a pressure of 10 cmH<sub>2</sub>O for 4 h with freshly prepared 4% paraformaldehyde in 0.1 M PBS,

pH 7.4. The lungs were removed, and lung volumes were measured by the volumetric displacement method (42). The fixed lungs were cryopreserved in 30% sucrose in 4% paraformaldehyde/0.1 M PBS, pH 7.4, at 4°C overnight, then placed in Tissue-Tek OCT compound (Sakura Finetek USA, Torrance, CA), frozen under liquid nitrogen, and stored at -70°C. Sections (3 µm) were cut at -22°C using a cryostat (Cryocut 1800, Reichert-Jung). The lungs were oriented so that the sections were sagittal, and the midsagittal section from each fetus was used for examination (31). We determined the effects of oligohydramnios on 1) the differentiation of distal pulmonary epithelium into type I and type II cells and 2) the relative amounts of parenchyma, nonparenchyma, and potential air space. Double indirect immunofluorescence was used to identify type I and type II cells on the same section. Cryosections were washed three times with 10 mM PBS, pH 7.4, containing 0.3% Triton X-100 and 1% BSA (PBS+); blocked with 10% normal goat serum (NGS)/PBS+ for 30 min at room temperature; and incubated in monoclonal primary antibodies to RTI<sub>40</sub> (culture supernatant diluted 1:1 with 10% NGS/PBS+) and RTII<sub>70</sub> (IgG fraction diluted 1:100 with 10% NGS/PBS+) simultaneously at 4°C overnight. Sections were washed five times with PBS+ and incubated sequentially in secondary antibody. All incubations and washes were performed at room temperature. RTI<sub>40</sub> was detected using biotinylated goat anti-mouse IgM (Zymed Laboratories, South San Francisco, CA) followed by Texas red-conjugated avidin (Molecular Probes, Eugene, OR), both diluted 1:500 in PBS+ for 15 min. RTII<sub>70</sub> was detected using fluorescein-conjugated rabbit anti-mouse IgG<sub>3</sub> (Cappell Organon Teknika, Durham, NC) diluted 1:200 in PBS+ for 30 min. The sections were counterstained with the nuclear dye 4,6-diamidino-2-phenylindole (DAPI; Sigma, 1 mg/ml) diluted 1:1,000 in PBS+ for 5 min to label all nuclei. After staining, lung sections were coverslipped using 1,4-diazabicyclo[2,2,2]octane (Sigma) and analyzed using a Leitz Orthoplan 2 microscope (Wetzlar, Germany) equipped with epifluorescence. For each lung, three images (phase contrast, DAPI, and RTI<sub>40</sub> + RTII<sub>70</sub>) of 30 randomly selected, nonoverlapping microscopic fields (×400) were acquired, through appropriate filter cubes, as digital images using a video camera (Optronics VI-470 CCD, Goleta, CA) and image analysis software (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). The acquired images were then saved on computer disks. We used all 30 fields for each fetus to calculate the variables described below.

**Measurement of air space perimeter.** We used differential immunofluorescent staining of RTI<sub>40</sub> (red) and RTII<sub>70</sub> (green) to calculate the relative perimeter of potential air spaces covered by type I cells and type II cells, respectively. We used Image-Pro Plus to analyze 30 fields for each fetus. For each field, we extracted the red image, used the Threshold tool to adjust the appearance to that of the original image, and then measured the total length of air space perimeter covered by RTI<sub>40</sub>. The process was then repeated on a green-extracted image to give a total length of air space perimeter covered by RTII<sub>70</sub>. There was very little overlap of the two images; areas of overlap involved <2% of the total length of RTI<sub>40</sub> and <5% of the total length of RTII<sub>70</sub>. For each field, we recorded the values of total length of RTI<sub>40</sub> (LRTI<sub>40</sub>), total length of RTII<sub>70</sub> (LRTII<sub>70</sub>), and the ratio LRTI<sub>40</sub>/LRTII<sub>70</sub>. We then calculated the average of each variable for each fetus. The accuracy of this method was >98% using lines of known length. The interobserver variability for the method was <2%.

**Measurement of parenchyma, nonparenchyma, and potential air space.** Nonparenchyma was defined as blood vessels, airways, septal tissue, and nonlung tissue or space. Paren-

chyma was defined as areas of gas-exchange tissue and potential air spaces as defined by the presence of immunofluorescence from RTI<sub>40</sub> and/or RTII<sub>70</sub> on the luminal surfaces. Luminal areas in these regions were the only areas counted as potential air spaces. Using Image-Pro Plus, we manually delineated areas of nonparenchyma in each microscopic field. These areas were measured and were subtracted from the overall area of the field. The remaining area was recorded as parenchyma. For parenchyma, we then measured areas of tissue and of potential air space. For each fetus, we calculated average values for nonparenchymal and parenchymal areas and expressed them as percentages of total area of the microscopic fields. In addition, for the parenchymal area, we calculated tissue area and potential air space area as percentages of the total parenchymal area. With figures of known area, the accuracy of the method was >97%.

**Statistical analyses.** Lung weights (wet and dry) were expressed in milligrams and, to correct for differences in fetal size, as a percentage of body weight. We used Student's paired *t*-test to compare variables from fetuses exposed to oligohydramnios with their controls, except for corticosterone measurements. Not all fetuses used for serum corticosterone measurements were paired by uterine position. Therefore, we used the unpaired *t*-test to compare corticosterone concentrations from oligohydramnios fetuses with controls. All statistical analyses were done using the computer program Statview (Abacus Concepts, Berkeley, CA). Data are presented as means  $\pm$  SD. *P* values of <0.05 were considered significant.

## RESULTS

To examine effects of decreased distension of the fetal lung, we produced oligohydramnios in fetal rats at 16 days of gestation by needle puncture of the uterine wall and fetal membranes of each uterine sac in one horn. Fetuses in corresponding positions in the opposite uterine horn served as controls. Fetuses were delivered at either 21 or 22 days of gestation.

Oligohydramnios significantly retarded growth of the fetal lungs and of the fetal body (Table 1). The magnitude of growth retardation was greater on the lungs than on the body. In oligohydramnios fetuses, the average deficit in body weight was <10%, whereas the deficit in lung weight was 25–30%. In addition, lung weights of oligohydramnios fetuses, expressed as a percentage of body weight, were lower than their controls by 20–24%. Therefore, oligohydramnios did produce pulmonary hypoplasia.

Fetal lung growth is dependent on lung distension (26, 29), and oligohydramnios retards fetal lung growth by causing less lung distension (12, 35, 40). Based on these considerations, we have assumed that, for oligohydramnios fetuses in which lung growth was not retarded, the procedure used to induce oligohydramnios (i.e., needle puncture of the uterus and fetal membranes) did not result in less lung distension. Because, as stated in our hypothesis, the aim of the current studies was to examine the effects of lower lung distension (and not oligohydramnios per se) on various indicators of fetal lung development, we have excluded from further analysis all pairs of fetal rats in which the lung weight of the oligohydramnios fetus was a greater percentage of body weight than the lungs of its corresponding control. This resulted in exclusion of three pairs of fetuses from two pregnant dams (out of a total of 102 pairs of fetuses from 56 dams).

Compared with control fetuses, lung water was significantly lower in oligohydramnios fetuses delivered both at 21 days (control  $7.26 \pm 0.46$ ; oligohydramnios  $6.64 \pm 0.66$  g H<sub>2</sub>O per g of dry lung,  $n = 10$ ,  $P < 0.005$ ) and at 22 days (control  $8.27 \pm 0.78$ , oligohydramnios  $7.45 \pm 0.83$  g H<sub>2</sub>O per g of dry lung,  $n = 11$ ,  $P < 0.002$ ). Table 2 shows data for DNA and protein. In oligohydramnios fetuses, the total amounts of DNA and protein in the lungs were less than controls. To correct for differences in lung water, we calculated lung DNA and protein concentrations per gram of dry lung weight, and both concentrations tended to be higher in oligohydramnios fetuses.

Corticosterone was measured in serum from 38 fetuses delivered at 21 and 22 days from four pregnant dams. Corticosterone concentrations were similar in the two experimental groups at both 21 days (control  $27.4 \pm 5.1$   $\mu$ g/dl, oligohydramnios  $29.1 \pm 5.5$   $\mu$ g/dl;  $n = 7$  for each) and at 22 days (control  $24.7 \pm 5.6$   $\mu$ g/dl, oligohydramnios  $21.4 \pm 4.1$   $\mu$ g/dl;  $n = 12$  for each).

Oligohydramnios did not significantly affect indicators of pulmonary surfactant. Lung concentrations of total lipids, phospholipids, and SatPC, expressed per milligram of dry lung weight, did not differ significantly between control and oligohydramnios fetuses delivered at either 21 days or 22 days (Table 3), although SatPC more than doubled from 21 days to 22

Table 1. *Effects of oligohydramnios on growth of the body and lungs in fetal rats*

Gestational Age/Group	BW, g	Wet Lung Weight		Dry Lung Weight	
		mg	%BW	mg	%BW
16–21 days ( $n = 43$ )					
Control	$3.769 \pm 0.358$	$126 \pm 15$	$3.34 \pm 0.28$	$15.2 \pm 1.8$	$0.40 \pm 0.03$
Oligohydramnios	$3.534 \pm 0.356^*$ (94%)	$95 \pm 13^*$ (75%)	$2.69 \pm 0.28^*$ (80%)	$12.5 \pm 1.7^*$ (82%)	$0.35 \pm 0.04^*$ (87%)
16–22 days ( $n = 47$ )					
Control	$5.106 \pm 0.739$	$145 \pm 25$	$2.84 \pm 0.35$	$15.8 \pm 2.8$	$0.31 \pm 0.04$
Oligohydramnios	$4.682 \pm 0.805^*$ (92%)	$102 \pm 25^*$ (70%)	$2.17 \pm 0.36^*$ (76%)	$12.2 \pm 3.0^*$ (77%)	$0.26 \pm 0.04^*$ (84%)

Data are means  $\pm$  SD;  $n$  = no. of pairs of fetal rats. Nos. in parentheses are % of corresponding control value. Gestational age, gestational age from onset of oligohydramnios to delivery of fetus; BW, fetal body weight; %BW, lung weight as a percentage of fetal body weight; \* $P < 0.001$  vs. corresponding control value.

Table 2. *Effects of oligohydramnios on DNA and protein in lungs of fetal rats*

Gestational Age/Group	Total Content in Lungs, mg		Concentration in Lungs, mg/g of dry lung wt	
	DNA	Protein	DNA	Protein
16–21 days ( <i>n</i> = 15)				
Control	0.94 ± 0.18	8.26 ± 1.11	58.1 ± 8.4 NS ( <i>P</i> = 0.06)	512.9 ± 35.2
Oligohydramnios	0.82 ± 0.17‡ (87%)	7.12 ± 1.02* (86%)	63.3 ± 10.4 (109%)	553.3 ± 59.3‡ (108%)
16–22 days ( <i>n</i> = 12)				
Control	0.89 ± 0.14 NS ( <i>P</i> = 0.09)	8.46 ± 1.08	53.9 ± 10.8	507.5 ± 58.6
Oligohydramnios	0.83 ± 0.08 (93%)	7.52 ± 0.90† (89%)	64.9 ± 6.7† (120%)	585.2 ± 52.2† (115%)

Data are means ± SD; *n* = no. of pairs of fetal rats. Nos. in parentheses are % of corresponding control value. Gestational age, gestational age from onset of oligohydramnios to delivery of fetus NS, not significant. \**P* < 0.001, †*P* < 0.005, ‡*P* < 0.05 vs. corresponding control value.

days in both control and oligohydramnios fetuses. Furthermore, there were no significant differences between oligohydramnios and control fetuses for mRNA for SP-A, -B, -C, and -D or for lung concentrations of the proteins SP-A and -B (Table 4).

To evaluate effects of decreased distension of the fetal lung (produced by oligohydramnios) on differentiation of the distal pulmonary epithelium, we measured RTI<sub>40</sub> and RTII<sub>70</sub>, proteins that are specific in lung to the luminal surfaces of alveolar type I and type II cells, respectively. We expressed the level of RTI<sub>40</sub> mRNA and of both proteins (RTI<sub>40</sub> and RTII<sub>70</sub>) as ratios of the level in the oligohydramnios fetus to its corresponding control. These results are shown in Fig. 1. There were no differences in RTII<sub>70</sub> between oligohydramnios and control fetuses delivered at either 21 or 22 days. In contrast, there were marked effects on type I cells as indicated by changes in RTI<sub>40</sub>. In oligohydramnios fetuses delivered at 21 days, RTI<sub>40</sub> mRNA and its protein were slightly lower than control, but the differences did not reach significance. However, in oligohydramnios fetuses delivered at 22 days, both RTI<sub>40</sub> mRNA and its protein were much lower than in controls, and the differences were significant.

For the five pairs of fetuses that were used for microscopic analysis, oligohydramnios did not affect body weight, but lung displacement volume (42) in oligohy-

dramnios fetuses was only 78.4% of control (Table 5). This magnitude of reduction in lung volume is similar to the magnitude of reduction in lung weight among other fetuses exposed to oligohydramnios (Table 1). On microscopic examination, lungs from oligohydramnios fetuses appeared to have smaller distal potential air spaces with more cellular septa (Fig. 2). Oligohydramnios did not significantly affect the proportion of total lung area occupied either by parenchyma (control 89 ± 4%, oligohydramnios 87 ± 6%) or by nonparenchyma (control 11 ± 4%, oligohydramnios 13 ± 6%). However, oligohydramnios did decrease the percentage of parenchymal area that was occupied by potential air spaces, and this difference nearly reached statistical significance (*P* = 0.056; Table 5).

To evaluate further the effects of oligohydramnios on differentiation of the distal pulmonary epithelium, we measured the total length of distal air space perimeter that was covered by type I cells (LRTI<sub>40</sub>) and by type II cells (LRTII<sub>70</sub>) and then calculated the ratio, LRTI<sub>40</sub>/LRTII<sub>70</sub>. In controls, LRTI<sub>40</sub>/LRTII<sub>70</sub> was 4.03 ± 0.33 (mean ± SD) and was reduced by 26% in oligohydramnios fetuses to 2.96 ± 0.58 (*P* < 0.05). Therefore, oligohydramnios significantly decreased the proportion

Table 3. *Effects of oligohydramnios on lipids and phospholipids in lungs of fetal rats*

Gestational Age/Group	Total Lipids	Phospholipids	SatPC
16–21 days ( <i>n</i> = 3)			
Control	176.7 ± 99.6	55.8 ± 5.0	5.6 ± 1.1
Oligohydramnios	117.7 ± 10.7	60.4 ± 3.5	7.2 ± 1.1
16–22 days ( <i>n</i> = 6)			
Control	164.0 ± 14.0	87.8 ± 9.7	19.4 ± 4.1
Oligohydramnios	176.0 ± 24.7	90.9 ± 12.4	19.8 ± 4.9

Values are means ± SD in mg/g of dry lung wt; *n* = no. of pairs of fetal rats. Gestational age, gestational age from onset of oligohydramnios to delivery of fetus; SatPC, saturated phosphatidylcholine. None of the oligohydramnios values differs significantly from its respective control value.

Table 4. *Effects of oligohydramnios on SP mRNA and on SP-A and -B*

Gestational Age at Delivery	SP mRNA			
	SP-A	SP-B	SP-C	SP-D
21 days ( <i>n</i> = 6)	1.01 ± 0.27	0.88 ± 0.14	0.83 ± 0.52	1.37 ± 0.92
22 days ( <i>n</i> = 6)	0.98 ± 0.49	0.87 ± 0.29	0.81 ± 0.30	1.10 ± 0.41
Gestational Age at Delivery	SPs			
	SP-A	SP-B		
21 days ( <i>n</i> = 6)	1.06 ± 0.29	1.05 ± 0.27		
22 days ( <i>n</i> = 7)	0.92 ± 0.24	1.21 ± 0.75		

Data are means ± SD; *n* = no. of pairs of fetal rats. All values are oligohydramnios values as a ratio of the corresponding control value. SP, surfactant protein. None of the oligohydramnios values differs significantly from its respective control value.

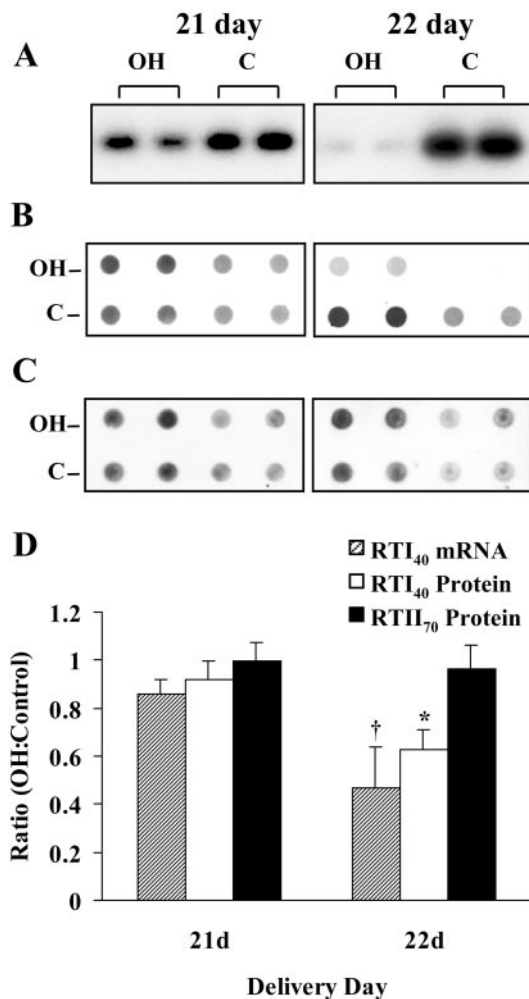


Fig. 1. Effects of oligohydramnios (OH) on RTI<sub>40</sub> mRNA and on the proteins RTI<sub>40</sub> and RTII<sub>70</sub> in lungs of fetal rats delivered at gestational days 21 (left) and 22 (right). **A:** RT-PCR of 250 ng of total RNA for RTI<sub>40</sub> mRNA ( $n = 6$  for both 21 and 22 days). **B:** Western dot blot of 5 and 2.5  $\mu$ g total protein, in duplicate, for RTI<sub>40</sub> protein ( $n = 22$  for 21 days;  $n = 13$  for 22 days). **C:** Western dot blot of 4 and 2  $\mu$ g total protein, in duplicate, for RTII<sub>70</sub> protein ( $n = 7$  for 21 days;  $n = 6$  for 22 days). **D:** graphic representation of A–C. Data shown are ratios of the oligohydramnios value to the corresponding control (C) value ( $\pm$ SE). \* $P < 0.001$ ,  $\dagger P < 0.05$  vs. corresponding control.

of distal air space perimeter covered by alveolar type I cells relative to alveolar type II cells, as illustrated in Fig. 2. In Fig. 2A (oligohydramnios), there is an apparent increase in type II cells (green) compared with the control (Fig. 2B); however, this appearance is, in fact, due to the decrease in perimeter of the potential air spaces covered by type I cells (red).

## DISCUSSION

Other investigators have reported that oligohydramnios retards growth of both the fetal body and lungs, but the effect is greater on the lungs as shown by the lower lung weight-to-body weight ratio in the fetuses subjected to oligohydramnios (7, 35). Our study shows similar results on fetal body and lung growth (Table 1). The probable explanation for the retarded lung growth

with oligohydramnios is less distension of the fetal lung due to a smaller volume of fluid in the potential airways and air spaces as described in fetal sheep (12, 40). This decrease in fluid volume is relatively rapid (40) and persists during oligohydramnios (12). Harding and Liggins (24) have shown that oligohydramnios causes changes in thoracic dimensions of fetal sheep that are consistent with decreased thoracic volume. On the basis of these studies and others cited in Refs. 26 and 29, it is reasonable to conclude that oligohydramnios causes a prolonged decrease in lung distension that retards lung growth. In the current study, the significantly lower lung water content in oligohydramnios fetuses and the lower amount of potential air space in oligohydramnios fetuses (Table 5) are consistent with this concept.

The lower lung contents of DNA and protein in the oligohydramnios fetuses (Table 2) are consistent with decreased lung growth. Of interest are the higher concentrations of DNA and protein in the lungs of oligohydramnios fetuses. These were calculated per gram of dry lung weight, so the lower amount of lung water in the oligohydramnios fetuses cannot explain the findings. A possible explanation is that, because DNA content per cell is constant, the higher concentration of DNA is due to smaller cell size, resulting from the lower degree of lung distension in oligohydramnios fetuses. However, this is speculative because we did not measure cell size.

To examine effects of oligohydramnios on surfactant, we measured SatPC (the main surface-active phospholipid in surfactant), SP-A and -B, as well as mRNA for SP-A, -B, -C, and -D. In previous studies in fetal sheep, we had found that lung water was decreased and that both SatPC and SP-A were increased after section of the cervical spinal cord, which abolished fetal breathing movements and caused pulmonary hypoplasia (28). Thus we expected in the current study that oligohydramnios would result in an increase in surfactant. However, oligohydramnios did not significantly affect any of the indicators of pulmonary surfactant (Tables 3 and 4). These results are consistent with those of Blachford and Thurlbeck (7), who also reported that oligohydramnios did not affect the lung concentration of SatPC in fetal rats. We do not know the reasons for the discrepancies between the results in fetal rats and

Table 5. Effects of oligohydramnios on lung morphology in fetal rats

	Fetal Weight, g	Lung Displacement Volume, ml	Potential Air Space, %parenchymal area
Control	5.539 $\pm$ 0.263	0.209 $\pm$ 0.013	65.9 $\pm$ 1.0
	NS		
Oligohydramnios	5.381 $\pm$ 0.186 (97%)	0.164 $\pm$ 0.022* (78%)	61.5 $\pm$ 3.2 <sup>†</sup> (93%)

Data are means  $\pm$  SD for 5 pairs of fetal rats; no. in parentheses are percentages of corresponding control. \* $P < 0.02$ ,  $\dagger P = 0.056$  vs. corresponding control.

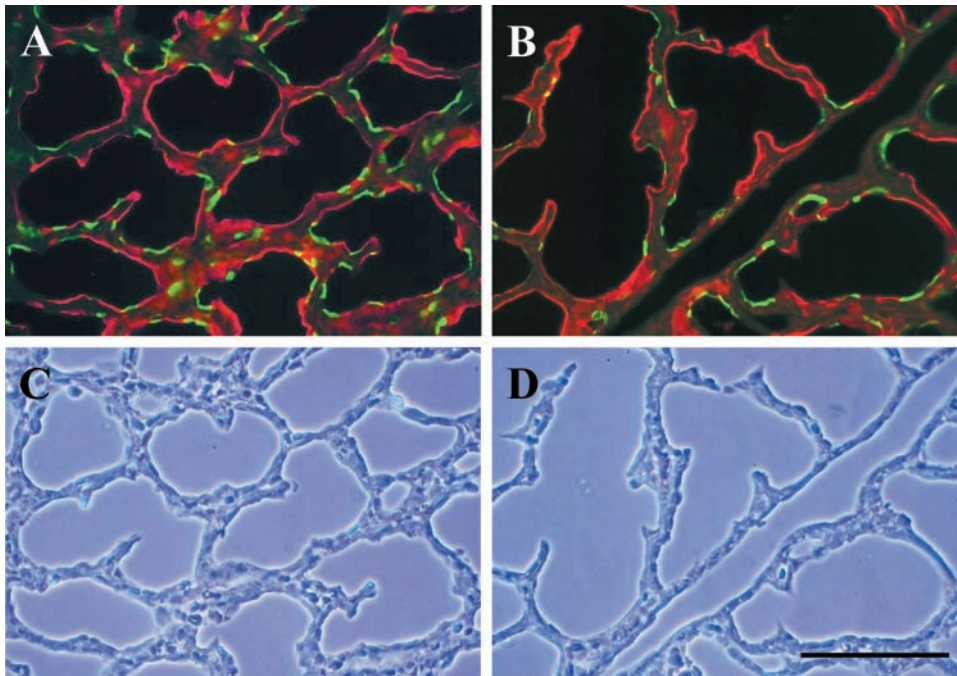


Fig. 2. Effects of oligohydramnios on lung morphology and epithelial differentiation in fetal rats. Sections of lung from a 22-day rat fetus, in which oligohydramnios was induced at *day 16* (A and C), and from its littermate control (B and D). A and B show differential immunofluorescent labeling of proteins specific to alveolar type I cells (RTI<sub>40</sub>, red) and type II cells (RTII<sub>70</sub>, green). C and D are phase-contrast images to show morphology. Lungs were fixed at an inflation pressure of 10 cmH<sub>2</sub>O. Oligohydramnios tended to decrease the percentage of parenchyma occupied by potential air spaces ( $P = 0.056$ ). This is exemplified in C (oligohydramnios), in which the potential air spaces appear smaller than in the control (D). Oligohydramnios also decreased the amount of distal air space perimeter covered by type I cells relative to type II cells ( $P < 0.01$ ). This is demonstrated in A (oligohydramnios), in which the decrease in air space perimeter covered by type I cells (red) results in an apparent increase in type II cells (green) compared with the control (B). Bar, 100  $\mu$ m.

those in fetal sheep. Possibilities include species differences, the method of inducing pulmonary hypoplasia (oligohydramnios vs. spinal cord section), the magnitude of change in distension, and the relative maturity of the lungs (10, 36). Previous investigators have shown that pulmonary surfactant in fetal rats is increased by both endogenous (45) and exogenous glucocorticoids (38). Therefore, the finding in the current study of similar plasma concentrations of corticosterone, the active glucocorticoid in rats, is consistent with the similar lung concentrations of the various indicators of surfactant in controls and in oligohydramnios fetuses.

Our results do not support our hypothesis that the lower degree of lung distension due to oligohydramnios would accelerate formation of pulmonary surfactant. In fact, with a decrease in lung size that appears to be due mostly to decreased formation of alveolar type I cells (as discussed below), one would expect relative increases in the concentrations of the components of pulmonary surfactant. Although there were increases in some components of surfactant in the oligohydramnios fetuses (e.g., SatPC at 21 days, Table 3; SP-B at 22 days, Table 4), these changes did not reach significance, and most surfactant components showed little or no change. Therefore, we cannot exclude a mild effect of oligohydramnios on type II cells.

Morphologically, oligohydramnios did not affect the proportion of lung occupied by parenchyma and by nonparenchyma, results similar to those reported by Blachford and Thurlbeck (7). However, in contrast to those authors, we found that the proportion of lung parenchyma occupied by potential air space was lower in oligohydramnios fetuses, a difference that almost reached statistical significance ( $P = 0.056$ ) (Table 5). Possible reasons for the differences between their re-

sults and ours include gestational age and the method of fixation of the lungs. Blachford and Thurlbeck (7) delivered fetuses at 21 days, removed the anterior portion of the thoracic cage, and then fixed the lungs at a transpulmonary pressure of 25 cmH<sub>2</sub>O for 24 h (7). We delivered the fetuses at 22 days and fixed the lungs at a pressure of 10 cmH<sub>2</sub>O for 4 h with the thoracic cage intact. Both the removal of the chest wall and the higher fixation pressure would tend to overdistend the lungs. In preliminary experiments, we found that fixation at 25 cmH<sub>2</sub>O produced lungs that appeared overdistended on microscopic examination. Therefore, we used the lower pressure and kept the fetal chest intact, a technique that resulted in uniform inflation of the lungs (Fig. 2) without the appearance of overdistension. The lower proportion of parenchyma occupied by potential air space in oligohydramnios fetuses in the current study is consistent with a lower volume of fluid in the potential air spaces and airways of the fetal lung, a previously described effect of oligohydramnios (12, 40).

We used RTI<sub>40</sub> and RTII<sub>70</sub>, proteins specific in the lung to the luminal surfaces of rat type I and type II pneumocytes, respectively (13, 20), to evaluate effects of decreased distension of the fetal lung (due to oligohydramnios) on pulmonary epithelial development. These results showed that the major effect was on type I cells. Both RTI<sub>40</sub> mRNA and its protein were slightly lower in oligohydramnios fetuses than in controls delivered at 21 days, and they were significantly decreased in those delivered at 22 days (Fig. 1). In contrast, there were no differences in RTII<sub>70</sub> at either 21 or 22 days. This apparent lack of effect on type II cells is consistent with our other findings of no changes in the various indicators of pulmonary surfactant (Tables 3 and 4). In the morphological studies, we exam-

ined the effects of oligohydramnios on the amount of air space perimeter covered by type I and by type II pneumocytes based on immunofluorescent staining of the apical membranes by antibodies to RTI<sub>40</sub> and RTII<sub>70</sub>, respectively. These results showed that, with oligohydramnios, the ratio LRTI<sub>40</sub>/LRTII<sub>70</sub> was 26% lower than in controls. Therefore, the major effects of a lower degree of fetal lung distension due to oligohydramnios was on type I cells. These effects included reduced expression of RTI<sub>40</sub> mRNA and its protein as well as diminished surface coverage by type I cells compared with type II cells in the distal potential air spaces. The effects of oligohydramnios on epithelial differentiation, although apparent at 21 days, were more pronounced at 22 days, a time when the prospective air spaces of the fetal rat lung are undergoing dilatation (9).

There is evidence also from *in vitro* studies that mechanical factors can influence alveolar epithelial differentiation. Alveolar type II cells cultured on plastic tend to lose features of type II cells, such as surfactant production (33), and acquire features of type I cells, such as expression of RTI<sub>40</sub> (14). Recently, Gutierrez and Dobbs (21) reported that mechanical contraction of cultured type II cells from adult rats inhibits expression of RTI<sub>40</sub>. Their *in vitro* results are concordant with our current results from rat fetuses *in vivo*. Conversely, Gutierrez and associates have shown that mechanical stretch increases RTI<sub>40</sub> mRNA in cultured type II alveolar cells (23) and in explants of fetal rat lung (22). Taken together, these studies support the concept that decreased distension of the lung favors expression of the type II cell phenotype, whereas increased lung distension has opposite effects (49).

In addition to effects on RTI<sub>40</sub> expression, Gutierrez and associates (21–23) found that stretch caused a reduction and contraction caused an increase in mRNA for SP-B and -C. In contrast, we found that oligohydramnios affected neither the mRNA for SP-A, -B, -C, or -D nor the proteins SP-A and -B (Table 4). Differing experimental conditions may have led to these disparate findings. Gutierrez and associates (21–23) used cultures of type II cells and of fetal lung explants, whereas the current studies were done in fetal rats *in vivo*. The duration of the mechanical stimulus *in vitro* (i.e., stretch or contraction) was 18 h (23), whereas in the current studies oligohydramnios was induced 5 or 6 days before birth. Also, the magnitude of mechanical stimulus (i.e., change in surface area) is likely to be different. Gutierrez and associates (23) changed surface area by 21%. Although we have no measurements of alveolar surface area in our studies, some assumptions can be made from our data. Lung water in oligohydramnios fetuses was 9% lower than in controls. If we assume that the volume of fluid in the potential airways and air spaces was also 9% lower in oligohydramnios fetuses and that the shape of the potential air spaces resembles a sphere, then the reduction in lung surface area would be only 5%, a much smaller mechanical stimulus than that applied during the *in vitro* experiments.

Changes in distension of the fetal lung affect the amount of stretch applied to the lung tissue and have diverse effects in the lung *in vivo*. The current studies show that decreased distension not only retards lung growth but also inhibits differentiation of type I cells. Previous studies have shown that increased lung distension in fetal sheep accelerates lung growth and decreases numbers of type II cells and surfactant (2, 11, 18, 28, 32, 37). The cellular mechanisms involved in the responses to changes in distension of the fetal lung have not been defined. However, it is likely that the cellular effects of mechanical stimuli are transduced through force-dependent changes in the cytoskeleton, a system based on tensegrity architecture (4, 27).

In summary, we have found that oligohydramnios, which reduces the normal distension of the fetal lung, not only retards lung growth but also inhibits expression of RTI<sub>40</sub> and decreases the relative surface area of the potential air spaces covered by type I cells compared with type II cells. On the basis of this significant inhibition of RTI<sub>40</sub> expression, as well as the absence of effect on the expression of RTII<sub>70</sub>, SatPC, SP-A and -B, and mRNA for all four surfactant proteins, we conclude that the major pulmonary effect of oligohydramnios is to inhibit formation of type I cells and that it has relatively little influence on type II cell function. From our experiments, we cannot distinguish whether oligohydramnios decreases the number or the size of type I cells. Resolution of this point will require detailed, quantitative morphological studies.

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