Experimental Fetal Tracheal Ligation Reverses the Structural and Physiological Effects of Pulmonary Hypoplasia in Congenital Diaphragmatic Hernia

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● **Infants with congenital diaphragmatic hernia (DH) and profound pulmonary hypoplasia are currently unsalvageable. The authors previously demonstrated that tracheal ligation (TL) accelerates fetal lung growth and reverses the pulmonary hypoplasia of fetal nephrectomy. The purpose of this study was to determine if the pulmonary hypoplasia of experimental DH could be similarly reversed and, if so, whether the resulting lungs would show better function than those of their DH counterparts. Eighteen fetal lambs were divided into three experimental groups of six animals each. In group 1, DH was created at 90 days' gestation. In group 2, DH was created at 90 days' gestation and TL performed during the same operation. Group 3 consisted of sham-operated controls. These animals were delivered near full-term, and their lungs analyzed by standard morphometric techniques. Ten additional fetal lambs were divided into two experimental groups of five animals each. In group 4, DH was created at 90 days' gestation. In group 5, DH was created at 90 days' gestation and TL performed 20 days later, at 110 days' gestation. These animals were pressure-ventilated via tracheostomy over a 2-hour period in which Pao_2, $Paco_2$, and compliance were measured. Intratracheal pressure (ITP) was measured at the time of delivery in all groups. Upon retrieval, DH animals had abdominal viscera in the chest and small lungs; in contrast, DH/TL animals had the herniated viscera reduced from the chest by enlarged lungs. DH/TL lungs showed markedly increased growth, with significant increases in lung volume:body weight ratio (LV:BW; $P = .0001$), alveolar surface area (ALV.SA; $P = .0001$), and alveolar number (ALV#) ($P = .0001$) when compared with those of the DH or control group. This growth was associated with a normal maturation pattern based on histological appearance, normal airspace fraction, and normal alveolar numerical density. ITP in the DH/TL group was increased when compared with that of DH and control animals ($P = .0001$). Total lung DNA and protein were both elevated in the DH/TL animals ($P = .0001$). However, the DNA:protein ratio remained normal, suggesting lung growth had occurred through cell proliferation, not by hypertrophy. When ventilated over a range of settings, DH/TL lungs were more compliant ($P = .0001$) and achieved higher Pao_2s ($P < .003$) and lower $Paco_2$s ($P = .0001$) than their DH counterparts. From these data, the authors conclude: (1) Experimental fetal DH produces hypoplastic lungs that are not capable of adequate gas exchange with conventional ventilation. (2) Fetal tracheal ligation is capable of reversing these effects and accelerating lung growth beyond even normal levels, while preserving the normal maturation process. (3) The mechanical and physiological consequences of TL in DH are reduction of the herniated abdominal viscera from the chest by enlarged lungs that are more compliant and more efficient at gas exchange than those from DH alone. (4) Fetal TL produces increased ITP, which may be responsible for the pulmonary growth observed.**

INDEX WORDS: Congenital diaphragmatic hernia, pulmonary hypoplasia; tracheal ligation, fetal lung growth.

PROFOUND pulmonary hypoplasia associated with congenital diaphragmatic hernia (CDH) continues to be a lethal problem. Although postnatal lung growth does occur in cases of CDH, none of the present pulmonary support modalities, including extracorporeal membrane oxygenation (ECMO) is capable of supporting these patients long enough for meaningful development to take place.[1] This has led some to advocate antenatal repair of CDH to allow a longer period of time for the hypoplastic lung to develop.

Several experimental animal models of pulmonary hypoplasia suggest that fetal lung growth in CDH can be manipulated. A model of CDH in fetal lambs, using intrathoracic balloon inflation to simulate herniated viscera, produced fatal pulmonary hypoplasia.[2] Subsequent "correction of the hernia" by balloon deflation allowed sufficient lung growth to assure 100% survival.[3] This was followed by in utero repair of surgically created fetal diaphragmatic hernia in lambs, also resulting in prevention of gross lung hypoplasia and in survival at birth.[4] Although these experimental models offered promise, early attempts at antenatal CDH repair in humans have met with limited success.[5]

To date, all attempts to improve pulmonary hypoplasia in CDH have been based on the concept of hernia repair to allow room for subsequent lung growth. However, we believe that a better understanding of the mechanisms responsible for fetal lung growth might allow alternative approaches.

Previous studies have established a link between lung growth and in utero lung liquid dynamics. In fetal sheep, chronic drainage of lung liquid causes

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pulmonary hypoplasia, whereas retention of lung liquid by tracheal ligation produces growth.[6-10] Maximal lung growth in the normal sheep fetus occurs between 112 and 124 days' gestation.[11] This time frame coincides with a period of significant elevation in intratracheal pressure.[12] These findings suggest that increased intratracheal pressure may play a role in normal fetal lung growth. We wished to determine whether this mechanism could be exploited to increase lung growth in systems known to ordinarily result in pulmonary hypoplasia. Our first model mimics the well-documented natural experiment of bilateral renal agenesis (Potter's syndrome), by employing bilateral fetal nephrectomy as the stimulus for pulmonary hypoplasia. This model eliminated fetal urine contribution to amniotic fluid volume and any renal modulation of pulmonary growth. In this model, tracheal ligation completely reversed the pulmonary hypoplasia seen in anephric sheep. Morphometric analysis showed normal alveolar maturation and pulmonary growth well beyond that of controls.[13] The current study was undertaken to determine if the pulmonary hypoplasia of CDH could also be influenced by fetal tracheal ligation and, if so, whether the resulting large lungs would demonstrate better physiological function than their CDH counterparts.

MATERIALS AND METHODS

Experimental Design

In phase I, 18 lambs were divided into three experimental groups of 6 animals each. In group 1, diaphragmatic hernia (DH) was created at 90 days' gestation (full term = 145 days). In group 2, DH was created at 90 days' gestation and tracheal ligation (TL) performed during the same operation. In group 3, sham-operated control animals underwent hysterotomy only. Animals in groups 1, 2, and 3 were delivered near full-term and sacrificed at birth for structural analysis of lung tissue.

In phase II, 10 additional fetal lambs were divided into two experimental groups of five animals each. In group 4, DH was created at 90 days' gestation. In group 5, DH was created at 90 days' gestation and TL performed 20 days later, at 110 days' gestation. Animals in groups 4 and 5 were also delivered near full-term and were resuscitated to evaluate lung function.

Fetal Surgical Manipulation

Time-dated pregnant ewes were anesthetized at 90 days' gestation and a left-sided diaphragmatic hernia was created surgically on the fetus as previously described.[4]

For animals also undergoing tracheal ligation, the forelimb and chest of the fetus were returned to the uterus, and the neck was exposed. The trachea was then isolated and doubly ligated with no.5 silk below the level of the larynx. Care was taken to avoid injury to the phrenic nerve and esophagus. This wound was not closed.

The fetal head was then returned to the uterus. Amniotic fluid that had been previously removed and kept at 37°C celsius was reinfused, and 500 mg cefazolin added to the amniotic cavity. If significant amounts of fluid were lost, warm normal saline solution was added as a replacement. The amniotic membranes and uterine wall were closed with a 90-mm Ethicon (Somerville, NJ) surgical stapler. The abdomen was closed with a no.2 nylon running fascial stitch. Subcutaneous tissues and skin were closed in layers. The following day, the ewe received Bicillin (Wyeth Laboratories, Inc, Philadelphia, PA) (2 million units intramuscularly) before returning to the farm.

Delivery

All fetuses were retrieved by repeat cesarean section at 135 days' gestation. Immediately after hysterotomy, intratracheal pressure (ITP) transducers were placed and ITP recorded in all groups. For control and DH animals, ITP was measured caudal to the larynx. For DH/TL animals, this measurement was performed caudal to the ligation point.

Lung Preparation

For groups 1, 2, and 3, respiratory movements were prevented by placing a surgical glove over the fetal head as it was delivered, and by immediate injection of a lethal dose of Somlethal Euthanasia solution (J.A. Webster, Inc, Sterling, MA) into the umbilical vein. Each lamb was then weighed and the chest opened through a median sternotomy. Lung liquid was aspirated and measured. The lungs were excised and weighed. Fresh tissue samples for DNA/protein analysis were taken from the right and left apical and diaphragmatic lobes. These specimens were placed in vials and snap frozen in liquid nitrogen. Subsequent lung preparation, as previously described,[13] consisted of inflation/fixation with a buffered glutaraldehyde solution at 25 cm H_2O pressure for approximately 2 hours. Sampling of fixed tissue was performed by taking 1-cm^3 samples from standard positions on the periphery of the right and left apical and diaphragmatic lobes. These were rinsed in buffer solution and postfixed in 1% osmium tetroxide for 2 hours. Each of the four specimens was washed, dehydrated, cut into 1-μm sections, and stained with toluidine blue.

Morphometric Techniques

Lung volumes were determined by water displacement of the inflation-fixed lung. Morphometric analysis within the intraacinar region of the lung was performed using a Zeiss (Zeiss, Germany) laboratory microscope, with a projection head engraved with a 42-point coherent test lattice,[14] at a magnification of 400×. The analysis included counts of test points falling on airspaces and alveolar wall tissue, and of intersections between alveolar surface and the test line system. An alveolus was defined as an airspace either wholly or partially enclosed by respiratory epithelium, with the remaining boundary formed by an imaginary line connecting the ends of two septae (Hu et al, 1987). Alveolar number was estimated by counting alveolar profiles within the test area. Alveolar surface area was estimated by linear intercept; alveolar numerical density was estimated by the method of Weibel and Gomez.[15] Forty fields were counted for each lung—10 fields for each of the right apical, right diaphragmatic, left apical, and left diaphragmatic lobes.

DNA/Protein Analysis

Determination of DNA content of the samples of snap-frozen fresh lung tissue was determined by methods previously described by Ceriotti,[16] as modified by Giles and Myers[17] and Johnson and Guthrie.[18] Protein content was determined by the method of Lowry.[19]

Neonatal Resuscitation

After delivery, lambs in groups 4 (DH) and 5 (DH/TL) underwent resuscitation. While still attached to the placental circulation, tracheostomy cannulae, internal jugular venous lines, and common carotid arterial lines were placed. The umbilical cord was clamped and divided, and the animals were anesthetized with intravenous fentanyl and paralyzed with pancuronium. After aspirating tracheal fluid, the animals were ventilated with a Healthdyne (Marietta, GA) 105, type 3A time-cycled pressure-limited ventilator. Umbilical artery catheters were also placed.

Ventilatory support was initiated at a rate of 30 breaths per minute (bpm), a peak inspiratory pressure (PIP) of 25 cm H_2O, a peak end-expiratory pressure (PEEP) of 3 cm H_2O, and an F_{IO_2} of 1.0. Support was adjusted in response to arterial blood gas results. Maximum values across the group were 70 bpm (rate), 45 cm H_2O (PIP), and 5 cm H_2O (PEEP), although each animal did not always require the maximal settings. Tidal volume and minute ventilation were continuously monitored with a Bear (Riverside, CA) neonatal volume monitor. Hemodynamics were closely monitored, and animals were treated with volume and vasopressors as necessary to maintain systolic blood pressure above 75 mm Hg. Preductal and postductal blood gases were obtained at 10-minute intervals. The animals were resuscitated for 1 to 2 hours, then killed.

Statistical Analysis

Statistical analysis was performed using analysis of variance (ANOVA) for all groups. Pairs within each group were compared by using posthoc testing with the Scheffe-f test at the 95% confidence limit. *P* values of less than .05 were considered significant.

RESULTS

Phase I

Gross Results

In all animals with DH (Group 1) the spleen, stomach, and small bowel were present in the left part of the chest, and the lungs were markedly reduced in size. In all animals with DH and simultaneous TL (group 2), herniated viscera were completely reduced from the chest by the enlarged lungs, which had grown through the diaphragmatic defect and into the abdominal cavity (Fig 1).

Histology

Histologically, DH lungs showed marked thickening of the alveolar walls, appearing structurally immature when compared with controls. In contrast, DH/TL lungs demonstrated structural patterns similar to those of controls, with normal appearing alveoli and thin alveolar septa (Fig 2).

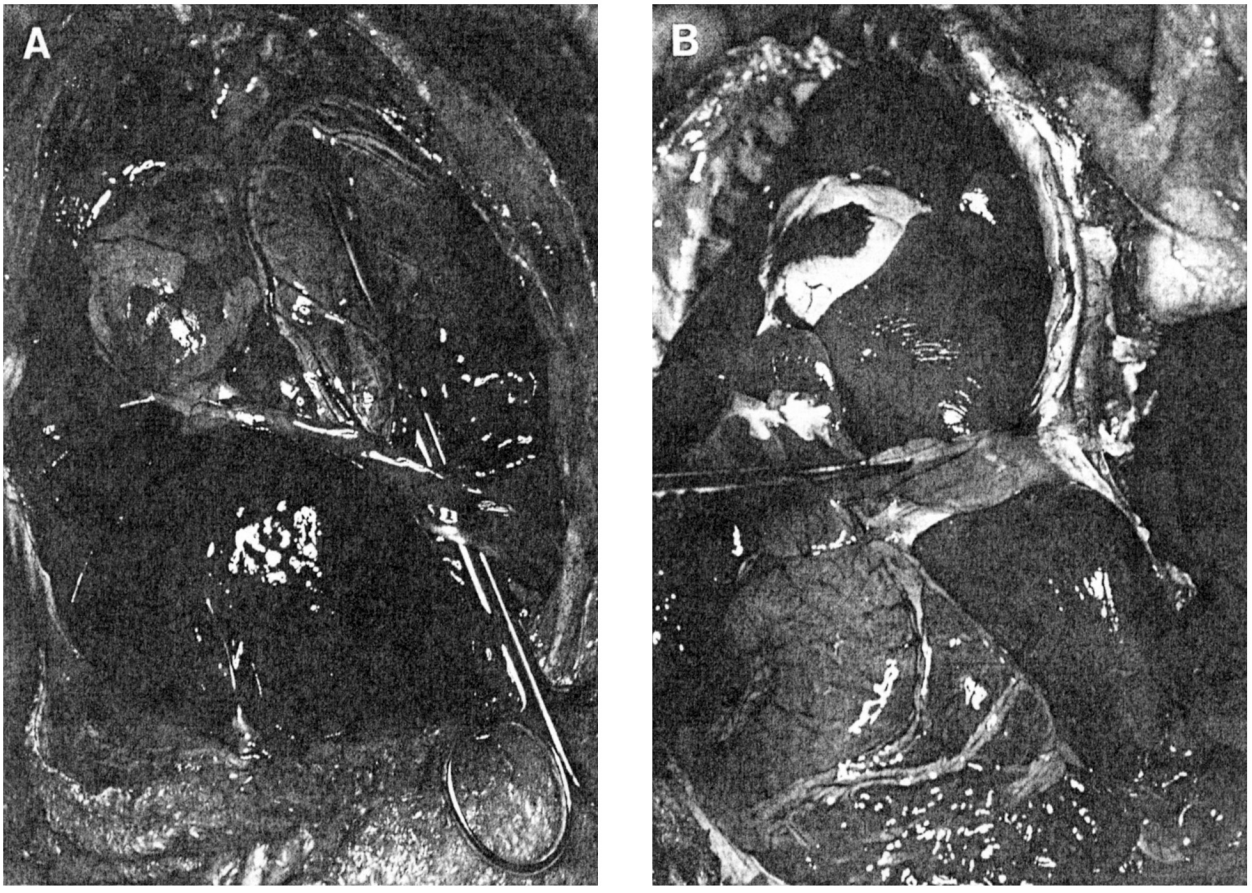

Fig 1. (A) DH animal, chest open via median sternotomy. Scissor passes through left diaphragmatic defect. Abdominal contents are present in left side of chest; lungs are small and not visible. (B) DH/TL animal, chest open via median sternotomy. Forceps are grasping the diaphragm. The enlarged lung has completely reduced the herniated viscera, and has grown into the abdominal cavity.

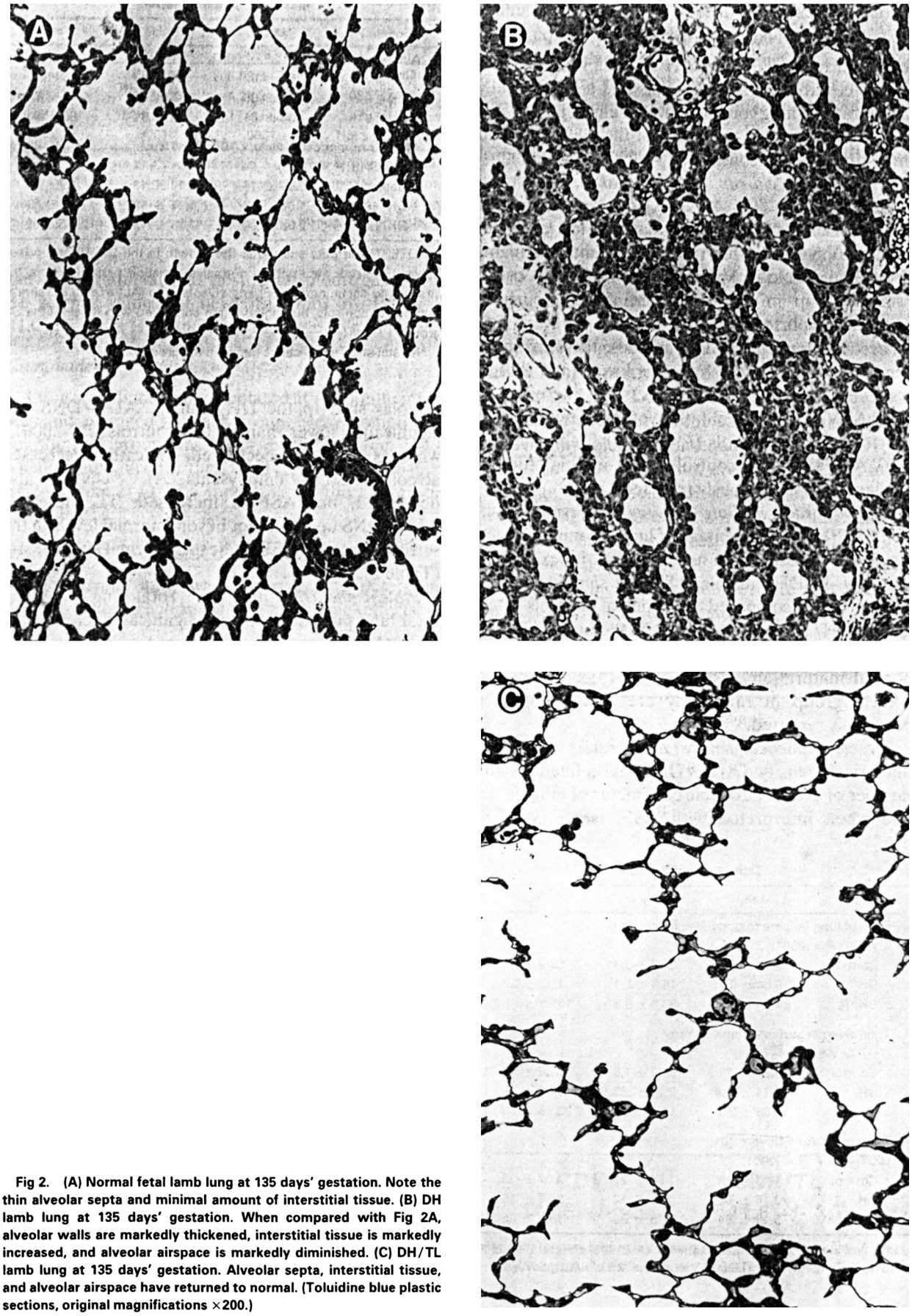

Fig 2. (A) Normal fetal lamb lung at 135 days' gestation. Note the thin alveolar septa and minimal amount of interstitial tissue. (B) DH lamb lung at 135 days' gestation. When compared with Fig 2A, alveolar walls are markedly thickened, interstitial tissue is markedly increased, and alveolar airspace is markedly diminished. (C) DH/TL lamb lung at 135 days' gestation. Alveolar septa, interstitial tissue, and alveolar airspace have returned to normal. (Toluidine blue plastic sections, original magnifications ×200.)

Morphometrics

Lung volume. When normalized to body weight, the mean lung volume of DH animals was less than that of controls. DH/TL animals had a dramatically higher ratio of lung volume to body weight (LV:BW)—approximately seven times that of the DH group and three times that of controls ($P = .0001$) (Table 1).

Alveolar surface area. In DH animals, total alveolar surface area (TOT.ALV.SA) was lower than that of controls. DH/TL animals had a 10-fold increase in TOT.ALV.SA over both DH and control animals ($P = .0001$; Table 1). Values for TOT.ALV.SA of the control group in this study are similar to those previously reported.[11]

Alveolar number. In DH animals, the total number of alveoli (TOT.ALV#) was lower than that of controls. In DH/TL animals, the increase in TOT.ALV# was 10-fold that of DH animals and fourfold that of controls ($P = .0001$) (Table 1). The TOT.ALV# of the control group in this study is similar to those previously reported.[9]

Airspace fraction (lung maturity). Airspace fraction (ASF) is the percentage of lung volume occupied by air, as opposed to tissue, and is an index of lung maturity. In DH animals, ASF was significantly decreased when compared with that of controls ($P = .0001$). DH/TL animals showed an increased ASF, up to but not exceeding normal levels, which indicates normal maturation. (Table 2). ASF values for the control group in this study are similar to those previously reported.[8,9]

Alveolar numerical density (alveolar size). Alveolar numerical density (ALV#DNS) is defined as the number of alveoli per cubic centimeter of lung tissue, and when interpreted with ASF, is an index of alveolar size. In the DH animals, ALV#DNS was significantly lower than that of controls ($P = .0001$), whereas ASF was decreased, indicating decreased alveolar size. In emphysema, ALV#DNS is also decreased, but ASF is increased. TL increased ALV#DNS up to but not beyond normal levels in the setting of normal ASF, indicating normal alveolar size (Table 2).

Table 1. Lung Growth

	Left	Right	Total
Ratio of lung volume to body weight (cm^3/kg) (LV:BW; $P = .0001$)			
Control	22.2 ± 2.2	31.7 ± 3.3	53.9 ± 5.5
DH	6.04 ± 0.7	16.3 ± 2.1	22.3 ± 2.8
DH/TL	75.6 ± 7.3	80.0 ± 5.3	155.5 ± 11.4
Total alveolar surface area × 10^4 cm^2 (TOT.ALV.SA; $P = .0001$)			
Control	3.94 ± .71	5.62 ± 1.0	9.56 ± 1.7
DH	0.74 ± .12	2.27 ± .33	3.01 ± .45
DH/TL	16.65 ± 2.3	17.3 ± 2.2	33.96 ± 4.3
Total alveolar number × 10^9 (TOT.ALV.#; $P = .0001$)			
Control	1.13 ± .13	1.54 ± .21	2.67 ± .35
DH	0.16 ± .03	0.51 ± .12	0.67 ± .15
DH/TL	5.13 ± .84	5.78 ± .96	10.9 ± 1.6

NOTE. Values are expressed as mean ± standard error of the mean. LV:BW, TOT.ALV.SA, and TOT.ALV.# are indices of lung growth.

Table 2. Lung Maturation

	Left	Right	Total
Airspace fraction (ASF; $P = .0003$)			
Control*	.62 ± .02	.63 ± .03	.63 ± .02
DH	.35 ± .08	.47 ± .05	.41 ± .07
DH/TL*	.66 ± .01	.65 ± .01	.66 ± .01
Alveolar numerical density × 10^7 alveoli/cm^3 (ALV#DNS; $P = .0001$)			
Control*	2.34 ± .17	2.17 ± .13	2.25 ± .14
DH	1.06 ± .11	1.27 ± .13	1.17 ± .11
DH/TL*	2.08 ± .09	2.25 ± .13	2.17 ± .08

NOTE. These data show that the growth in the DH/TL group (see Table I) is associated with a normal maturation pattern, where the airspace-to-tissue ratio (ASF) and alveolar size (ALV#DNS) are similar to those of controls, but higher when compared with those of the DH group.

*No statistical significance between the groups.

Protein and DNA analysis. Total lung DNA and total lung protein were both significantly elevated in the DH/TL group when compared with those of the DH and control groups ($P = .0001$). However, the DNA:protein ratio was similar in all three groups, suggesting that lung growth had occurred through cell proliferation, not through hypertrophy (Table 3).

Intratracheal pressure/lung liquid volume. On delivery, intratracheal pressure (ITP) in the DH and control groups was 0 mm Hg, versus 6.14 ± 0.55 mm Hg in the DH/TL group ($P = .0001$). Mean lung liquid volume in the DH group was 4.0 ± 0.71 mL versus 256.7 ± 28.9 mL in the DH/TL group, and 12.0 ± 2.3 mL in controls ($P = .0001$). In all groups, lung liquid was clear and transluscent.

Phase II

Physiological Studies

All animals in group 4 (DH alone) and group 5 (DH/TL) were delivered near full-term, and were resuscitated as previously described. The highest Pao_2 and the lowest $Paco_2$ attained were averaged for each group. The DH animals exhibited poor gas exchange, with a mean high Pao_2 of 51.0 ± 13.7 mm Hg and a mean low $Paco_2$ of 143.6 ± 9.9 mm Hg. DH/TL animals had improved function, achieving a mean high Pao_2 of 360.4 ± 71.5 mm Hg ($P = .003$) and a mean low $Paco_2$ of 41.6 ± 3.7 mm Hg ($P = .0001$). There were no significant differences

Table 3. DNA and Protein

	Lung DNA (g)	Lung Protein (g)	DNA:Protein Ratio
Control	.43 ± .07	5.69 ± .84	.076 ± .004
DH	.33 ± .33	4.25 ± .47	.080 ± .005
DH/TL	1.34 ± .13	17.8 ± .89	.075 ± .004
P value	.0001	.0001	Not significant

NOTE. Values are expressed as mean ± standard error of the mean. In the DH/TL group, significant increases in total lung DNA and total lung protein, with no change in the DNA/protein ratio, suggest that lung growth occurred through cell proliferation, not through hypertrophy.

between preductal and postductal blood gases (Fig 3). Normal values for newborn lambs are $PaCO_2$ of 41.0 ± 5.0 mm Hg[20] and PaO_2 of 259 ± 38 mm Hg.[21]

Compliance

Lung compliance was calculated at 10-minute intervals and averaged for each group. Mean compliance for the DH group was 0.184 ± .036 $cm^3/cm\ H_2O$. Mean compliance for the DH/TL group was 0.646 ± .062 $cm^3/cm\ H_2O$—3.5 times that of the DH group (P = .0001).

DISCUSSION

The current study was undertaken to determine whether the structural and physiologic effects of pulmonary hypoplasia in fetal diaphragmatic hernia (DH) could be reversed by tracheal ligation (TL).

Our data demonstrate seven principle findings. (1) Experimental fetal DH produces hypoplastic lungs that are not capable of adequate gas exchange with conventional ventilation. (2) Fetal TL in DH is capable of reversing these effects by accelerating lung growth beyond even normal levels, based on alveolar number, alveolar surface area, and the ratio of lung volume to body weight. (3) This growth is associated with a normal maturation pattern as evidenced by histological appearance, airspace fraction, and alveolar numerical density determinations. (4) Based on DNA:protein ratios, it appears that cell size is normal and that lung growth is achieved by cell proliferation rather than hypertrophy. (5) The mechanical consequence of TL in DH is reduction of the herniated abdominal viscera from the chest by enlarged lungs. (6) The physiological consequences of TL in DH are more compliant lungs that are more efficient at gas exchange than their DH counterparts. (7) Fetal tracheal ligation produces increased intratracheal pressure, which may be responsible for the pulmonary growth observed.

These findings clearly demonstrate that fetal tracheal ligation can accelerate lung growth beyond

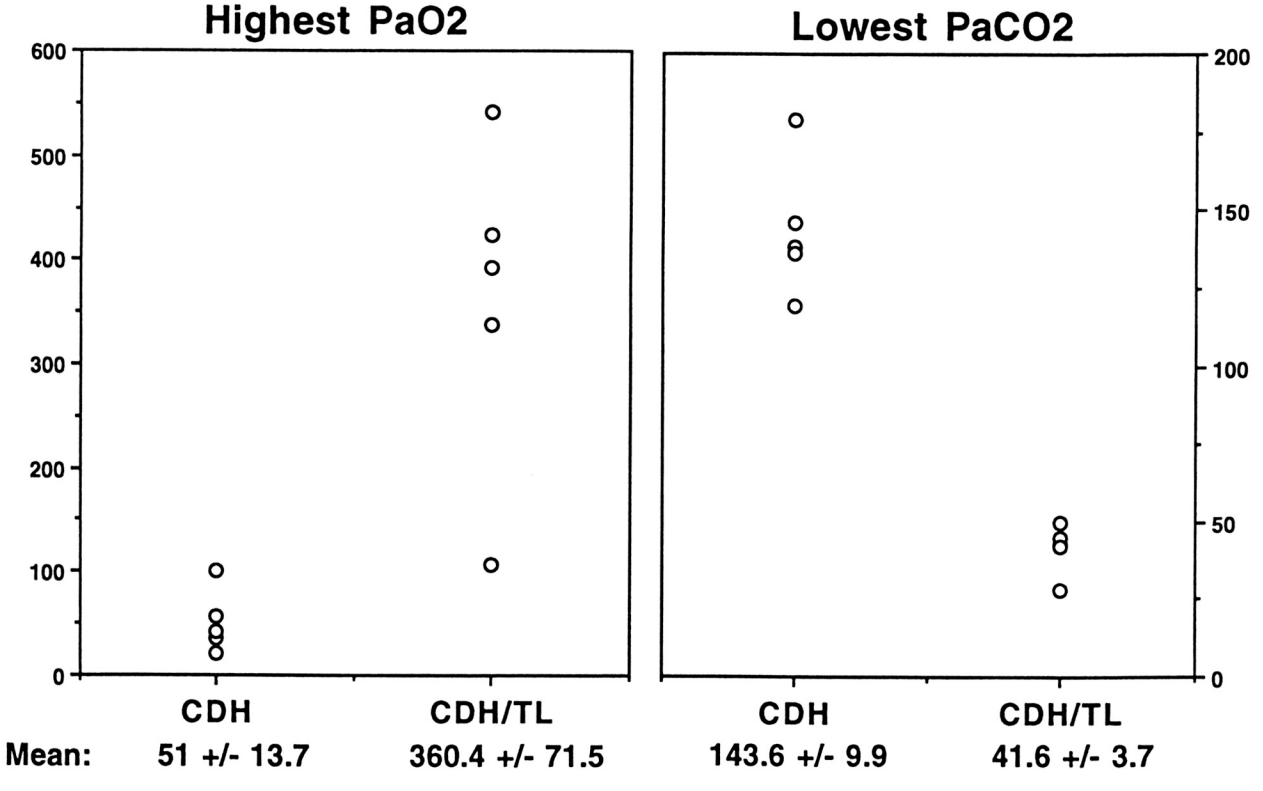

Fig 3. Values for individual animals are indicated by open circles. Mean values ± standard error of the mean are reported in the text. These data show significantly improved ventilation ($PaCO_2$), (P = .0001) and oxygenation (PaO_2), (P = .003) in the DH/TL group.

control levels, while preserving the normal maturation process. Data from our studies and those of others demonstrate enhanced lung growth with fetal tracheal ligation in normal lambs[6-8] and in those in which pulmonary hypoplasia had been previously induced.[10,13]

Several morphometric parameters merit special attention. LV:BW, TOT.ALV.SA, and TOT.ALV# correlate directly with lung growth, and in this study demonstrate markedly increased lung growth in the DH/TL group over that of DH and control animals. Lung maturation is determined by histological appearance, ASF, and ALV#DNS. DH/TL animals had normal histological appearance, ASF similar to controls, and ALV#DNS also similar to controls. Taken together, these results indicate a preservation of the normal template for pulmonary growth.

Although the specific mechanisms responsible for lung growth are unknown, we believe increased intrabronchial pressure may be involved. The ITP values of 6 to 7 mm Hg consistently found in the DH/TL group, are well above the 1.8 to 2.0-mm values for normal fetal lambs of similar gestational age in utero.[22,23] Studies by Vilos and Liggins,[22] Fewell and Johnson,[23] and Harding et al[24] suggest that outflow of lung liquid is normally impeded by the upper airway, giving rise to elevated pressures within the trachea. Docimo et al also showed that maximal lung growth in the normal sheep fetus occurs between 112 and 124 days' gestation,[11] which coincides with a period of significant elevation in intratracheal pressure as shown by Dickson and Harding.[12] Conversely, chronic drainage of fetal lung liquid with decreased ITP leads to pulmonary hypoplasia.[8,9] All these data support our hypothesis that tracheal ligation corrects pulmonary hypoplasia by enhancing normal mechanisms of fetal lung growth.

If increased intrabronchial pressure is involved in lung growth, what are the consequences of these changes in pressure? It is known that activation of pulmonary stretch receptors by increased ITP inhibits fetal breathing movements by increasing afferent activity in the vagus nerve.[25] This demonstrates that increased ITP and pulmonary "stretch" are already recognized in fetal lambs. It is also known that small pressure changes produce large volume changes in liquid-filled lungs of fetal lambs that are near full-term.[7] This is consistent with our observation that airway distending pressures of 4.0 to 5.0 mm Hg above normal do produce appreciable increases in airway lung liquid volumes. We believe this volume increase may translate into tension on the cells lining the airways. Several studies in vivo and in vitro have shown that cells respond to mechanical tension by increased mitosis and uptake of [^{3}H] thymidine.[26-28] That this is occurring in the current study is supported by the stable DNA:protein ratios, which suggest that lung growth is achieved by cell proliferation rather than hypertrophy. This hypothesis is further supported by similar findings demonstrated in other organ systems, such as vascular smooth muscle[29] and bladder.[30] In fact, soft tissue growth seen with tissue expanders, and pronounced growth of gastrointestinal tissue proximal to intestinal atresias, may be examples of a ubiquitous mechanism for growth.

We hypothesize that mechanical alveolar distension may promote pulmonary growth biochemically, through elaboration of local or systemic growth factor(s). In our model, concentration of local factors might be further increased by the delayed lung liquid drainage caused by tracheal ligation. On a molecular level, alveolar distension may involve alteration in the regulation of particular gene sequences. These are areas for future investigation, which we are pursuing.

Tracheal ligation, in addition to correcting the structural effects of pulmonary hypoplasia in DH, also markedly improved the lungs' capacity for gas exchange. However, this effect was achieved by a change in technique. Given our observation that performing TL at 90 days' gestation caused the left lung to grow through the hernia defect and into the abdomen (Fig 1), we had concerns about potential mechanical problems in ventilating this intraabdominal portion of the lung. We believed it was necessary to attenuate this explosive growth if ventilation was to be successful. This was achieved by performing TL at 110 days' gestation—a timepoint selected to coincide with data indicating an increased ITP at this gestational age in normal fetal lambs.[12] In this group of experimental animals, a positive pressure gradient of 6 to 7 mm Hg was present at delivery. The herniated viscera were again completely reduced by enlarged lungs. However, the lungs were contained within the thorax. While we are just beginning detailed studies of the pulmonary vasculature of these animals, it is encouraging that they function so well physiologically.

It is also intriguing that current postnatal pulmonary management of CDH includes stimuli that contradict the mechanisms of lung growth suggested by this study. Intubation and frequent suctioning of lung liquid would be expected to cause pulmonary hypoplasia in fetal lambs. Some studies even suggest that subsequent alveolar development in hypoplastic lungs is actually impaired by mechanical ventilation.[31]

These interesting findings may warrant reevaluation of the clinical treatment of CDH.

Before pursuing clinical studies, it will be important to determine in the laboratory whether tracheal ligation can be combined with ECMO support to accelerate postnatal lung growth. We have already demonstrated that lung growth does occur postnatally in CDH patients; however, the rate is too slow to improve lung function within the time limits of present ECMO support.[1] The results of the present study show that during a 25-day period (TL at 110 days' gestation; delivery at 135 days' gestation), lung growth was sufficient to reduce the DH and improve physiological function. If TL could produce a similar rate of growth postnatally, ECMO could support these infants during the process.

If it is determined that intervention must take place in the fetal period, tracheal occlusion may be easily adapted to a laparoscopic approach. Occlusion of the airway by an endoscopically delivered balloon apparatus or by external ligation of the fetal trachea with a surgical clip should result in lower morbidity than do present antenatal techniques. Pilot studies in "fetoscopic surgery" have already been reported,[32] and we have been successful in accessing the airway of a 95-day-gestation fetal sheep using laparoscopic equipment.

Additional understanding of the physiology and mechanisms of this manipulation are clearly necessary before it is attempted in the human fetus. However, present CDH treatment modalities that emphasize hernia repair and passive lung growth are failing. The lessons learned from these tracheal ligation studies offer insight into heretofore unrecognized mechanisms of fetal lung growth, which may alter our thinking about the management of this disease. Focusing initial CDH therapy on active promotion of lung growth by exploiting a mechanism that may be part of normal pulmonary development offers some hope of improved outcome for patients with this condition.

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Discussion

N.S. Adzick (San Francisco, CA): The fetal lung produces lung fluid under continuous positive airway pressure (CPAP). Fetal CPAP is normally about 3 cm H_2O, and this pressure within the developing airways is crucial for normal fetal lung growth. Dr DiFiore and his colleagues have shown that fetal CPAP can be increased by prenatal tracheal ligation, which expands the fetal lungs like a water balloon. In a lamb model of fetal diaphragmatic hernia, the remarkable results are that the lungs are much bigger than those of controls, the intestines are reduced from the chest into the abdomen, and most importantly, these lungs work well at birth.

One of our research fellows, Dr Marc Hedrick, has shown the exact same results in a fetal lamb CDH model by merely occluding the trachea during the last 2 weeks of gestation. We have learned from these experiments that plugging the fetal trachea for a very short period can lead to tremendous acceleration of fetal lung growth.

(SLIDE) This slide shows fetal lung growth schematically, under various circumstances. Fetuses with severe diaphragmatic hernia die because their lungs are too small. Repair of CDH before birth can lead to sufficient fetal lung growth for neonatal survival if the fetus remains inside the mother for a few weeks postoperatively, but the lung size is still less than normal at birth. Tracheal occlusion before birth results in lung size of even bigger than normal.

I have questions in three specific areas. First, how does this process work? What is the underlying biology? Presumably the increased fetal airway pressure leads to increased pulmonary stretch, which causes increased cell proliferation, and this process in turn is likely mediated by specific peptide growth factors. Have you measured growth factor levels in fetal lung fluid or examined growth factor mRNA expression in fetal lung tissue?

Second, is the same approach of increasing airway pressure applicable immediately *after* birth? For instance, can we help babies with severe pulmonary hypoplasia on ECMO using liquid ventilation at a particular pressure to accelerate neonatal lung growth in a week or two?

Finally, how would you implement this new therapeutic strategy to treat pulmonary hypoplasia *before* birth? We have developed a fetal tracheostomy device that has a one-way on-off valve that can be controlled outside the mother to reversibly occlude the fetal trachea. In the future, this device may be placed fetoscopically to treat fetuses with either severe oligohydramnios-induced pulmonary hypoplasia or severe diaphragmatic hernia.

T.C. Moore (Palos Verdes Estates, CA): This certainly is a fascinating study, and I think one of really immense potential clinical importance. I think hopefully we may see some real breakthroughs in the management of this dreadful problem in the newborn.

This report, indicating that fetal tracheal ligation reverses or prevents structural and physiological effects of pulmonary hypoplasia in congenital diaphragmatic hernia, suggests that this so-called hypoplasia may be acquired and possibly on an inflammatory basis related to aspiration of amniotic fluid. The coming poster of Patricia Donahoe and associates (to be presented here) also addresses the same issue of correction of this hypoplasia/immaturity problem in congenital diaphragmatic hernia by antenatal treatment with antiinflammatory glucocorticoids. Of particular interest are the findings from three major studies (published in 1990) by Moncada and associ-

ates of Greater London; the authors describe the inhibition of endotoxin-induced nitric oxide synthesis by porcine vascular endothelial cells through an inducible calcium-independent nitric oxide synthase mechanism. This is the mediator mechanism of major inflammation-producing molecules such as serotonin, bradykinin, histamine, substance P, and calcitonin gene-related peptide.

Fetoscopic insertion of an intratracheal plug, as Dr Adzick has mentioned, is perhaps worthy of consideration in addition to glucocorticoid administration.

J.W. DiFiore (response): I would like to thank Dr Adzick and Dr Moore for their insightful comments.

Dr Adzick, first in response to your question about growth factors, we have begun analysis of the lung liquid and amniotic fluid for a wide variety of growth factors. As far as your question regarding the potential mechanism of this phenomenon, I believe the increases in intratracheal pressure we observed may be the stimulus for elaboration of local or systemic growth factors. In addition, there may be a growth factor present in the lung liquid that, because of the tracheal ligation, is retained in the lung tissue in a higher concentration than would normally occur.

As far as your question regarding expression of peptides, we are presently evaluating the lung tissue for mRNA expression, using a differential display technique to evaluate several peptides simultaneously.

Regarding your question on the postnatal application of this phenomenon, we have already performed one experiment addressing this. We took one fetal lamb, created a hernia at 90 days' gestation and, on delivery, ligated the trachea while the animal was still attached to the placental circulation. We immediately placed the animal on ECMO to see if the lung would grow postnatally. However, no postnatal lung growth was observed. We postulate that growth did not occur because of loss of postitive intratracheal pressure, caused by absorption of the lung liquid by the lung postnatally. The next step we are pursuing involves providing the intratracheal pressure by a column of fluid that is nonabsorbable by the newborn sheep lung.

In regard to your final question concerning the clinical application in humans, although there is potential here for clinical intervention ultimately, it is clear that an extensive amount of work still needs to be done beforehand. First is vascular morphometric analysis of the lungs to determine the size and distribution of their vessels. Second is to determine an analysis of survival, and whether or not animals that have had tracheal ligation will be able to survive and breathe spontaneously. Third is determination of the effect of tracheal ligation on other organs, in particular the cardiac system. If these studies deem that this is appropriate for humans, the actual technique would probably involve a laparoscopic approach, which may cause less of an insult to the human uterus and hopefully obviate some of the present problems with preterm labor and fetal surgery. Another type of treatment may be the administration of pulmonary growth factors to the fetus in utero, if any are found.