Experimental Fetal Tracheal Ligation and Congenital Diaphragmatic Hernia: A Pulmonary Vascular Morphometric Analysis

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● **The authors have previously shown that fetal tracheal ligation (TL) reverses the pulmonary hypoplasia in experimental diaphragmatic hernia (DH) by accelerating fetal alveolar growth. The purpose of this study was to determine if growth of the accompanying macroscopic and microscopic pulmonary vasculature is also accelerated. Eighteen fetal lambs were divided into three experimental groups: diaphragmatic hernia (DH), DH and simultaneous tracheal ligation (DH/TL), and sham-operated controls (C). Animals were delivered near term, the lungs retrieved, and pulmonary capillary growth (5 to 50 μm in diameter) evaluated by standard morphometric techniques. Capillary ultrastructure was evaluated by electron microscopy. Nine additional fetal lambs of the same gestational age were equally divided into the same three groups and their lungs analyzed by pulmonary arteriography for evaluation of large vessel growth (<100-μm diameter). Computer digital analysis of angiogram lung slices showed that the total area of large vessels was increased in DH/TL lungs when compared with DH lungs and decreased in DH lungs when compared with C lungs ($P = .003$); however, the ratio of large vessel area per unit of lung area was similar in all groups. Microscopic morphometry of the capillary bed showed that the total number of capillaries was increased in DH/TL lungs over both DH and C lungs ($P = .0001$); however, the number of capillaries per alveolus (cap/alv) was similar in all groups. In DH/TL lungs, electron microscopy showed normal capillary wall structure and normal thickness of the capillary-alveolar interface, whereas in DH lungs, capillary structure was abnormal and the capillary-alveolar interface was thickened. DH animals had three times as many fully muscularized vessels as DH/TL and twice as many fully muscularized vessels as C animals ($P = .0001$). Furthermore, in DH lungs, 29% of vessels less than 100 μm in diameter were fully muscularized, whereas in DH/TL and C lungs, there were no fully muscularized vessels less than 100 μm in diameter ($P = .0009$). From these data the authors conclude the following: (1) Experimental fetal DH results in hypoplasia of both large pulmonary vessels and the capillary bed. (2) Fetal tracheal ligation is capable of reversing these effects by accelerating both large vessel and capillary growth based on total large vessel area and total capillary number. (3) With TL, large vessel growth remains proportional to overall lung growth based on normal values for large vessel area per unit of lung area, and capillary growth remains proportional to alveolar growth based on normal values for cap/alv and cap/cm^2 lung tissue. (4) Experimental fetal DH results in increased muscularization of pulmonary arterial vessels as evidenced by an increased overall percentage of muscular arteries and by extension of muscle into normally nonmuscular vessels. (5) Fetal tracheal ligation reverses the increased muscularization of pulmonary arteries seen in experimental fetal DH. (6) By electron microscopy, capillary wall ultrastructure and the capillary-alveolar interface are abnormal in DH lungs and normal in DH/TL lungs. The authors speculate that tracheal ligation exerts its effects by enhancing normal mechanisms of pulmonary development, thereby preserving the normal template for both pulmonary arterial and alveolar growth.**

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

WE HAVE PREVIOUSLY shown that experimental fetal tracheal ligation accelerates fetal lung growth, reverses the alveolar pulmonary hypoplasia seen in surgically created fetal diaphragmatic hernia (DH), and restores physiological function of the DH lungs to normal.[1-2] Although alveolar growth in these studies was quantified by detailed morphometric analysis, concurrent growth of the pulmonary arterial tree was only inferred based on improvements in gas exchange in the animals with both diaphragmatic hernia and tracheal ligation (DH/TL). If we are to confirm our hypothesis that tracheal ligation (TL) exploits normal mechanisms of lung growth and if the technique of tracheal occlusion is to have any clinical utility, it will first be necessary to determine the effect of TL on vascular as well as alveolar lung growth. The purpose of the current study, therefore, is to determine quantitatively whether the accelerated alveolar growth seen with fetal tracheal ligation is accompanied by a proportional acceleration in growth of the pulmonary vasculature.

MATERIALS AND METHODS

Experimental Design

Eighteen fetal lambs were divided into three experimental groups of six animals each. In group 1, DH was created at 90 days' gestation (term = 145 days). In group 2, DH was created at 90 days' gestation, and TL was performed during the same operation. In group 3, sham-operated control animals underwent hysterotomy only. Animals were delivered near term by repeat hysterotomy and killed at birth for morphometric analysis of pulmonary capillaries (5 to 50 μm in external diameter). Capillary wall ultrastructure and

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the capillary-alveolar interface were also evaluated by electron microscopy. Nine additional fetal lambs of the same gestational age were equally divided into the same three groups, and their lungs were analyzed by pulmonary arteriography for evaluation of large vessel growth (> 100 μm in external diameter).

Fetal Surgical Manipulation

Time-dated pregnant ewes at 90 days' gestation were anesthetized, and a left-sided diaphragmatic hernia was surigcally created in the fetus as previously described.[3] For those animals also undergoing tracheal ligation, the trachea was isolated and doubly ligated with no. 5 silk below the level of the larynx. All fetuses were delivered by repeat cesarean section at 135 days' gestation, and the lungs were retrieved.[2]

Large Vessel Analysis

For the nine animals undergoing pulmonary arteriography, lungs were stored at −80°C immediately after retrieval. Before pulmonary arterial injection, lungs were thawed overnight at room temperature and placed in a 37°C water bath for 1 hour. The pulmonary artery was injected with a quick-setting barium sulfate/gelatin mixture at 60°C and 100 cm H_2O pressure until a characteristic white coloration was noted at the pleural surface.[4-5] The pulmonary artery cannula was then occluded and the lungs' inflation fixed via the trachea with a buffered glutaraldehyde solution at 25 cm H_2O for 2 hours.

The barium perfused/inflation fixed lungs were sliced into coronal sections 4 mm thick. Radiographs of each slice were prepared on Kodirex nonscreen film (Eastman Kodak, Rochester, NY), using 45 keV and 15 mA for 1.5 seconds at a standard tube to target distance of 50 cm. Films were developed by the Kodak X-omat automatic process. 35-mm slides were made of each radiograph using a standard focal length. These were converted to digital images with a Kodak digital camera and transferred to a Macintosh computer for analysis using the National Institutes of Health IMAGE software package. This allowed quantitative evaluation of total lung area, total large vessel area, and total large vessel area per unit of lung area.

Capillary Vessel Analysis

Preparation of lung tissue and subsequent morphometric analysis within the intra-acinar region of the lung were performed using light microscopy as previously described.[2] Forty fields were counted for each lung: 10 fields for each of the right apical, right diaphragmatic, left apical, and left diaphragmatic lobes. The number of capillaries per alveolus (cap/alv) was determined by counting capillaries and alveolar profiles within the test area. The total number of capillaries (tot cap #) was determined by multiplying the number of capillaries per alveolus (cap/alv) by the total alveolar number. The number of capillaries per cm^2 lung tissue (cap/cm^2) was determined by multiplying the number of capillaries per alveolus (cap/alv) by the alveolar numerical density (no. of alveoli/cm^2 lung tissue).

Tissue for electron microscopic analysis was fixed and embedded as previously described.[2] Silver sections were cut with a LKB ultramicrotome (LKB, Sweden), collected on 200-mesh copper grids and stained with uranyl acetate and lead citrate. Samples were viewed and micrographs obtained with a Zeiss EM10 transmission electron microscope (Zeiss, Germany).

Arterial Muscularization Analysis

The barium injected/inflation fixed tissue was stained with Miller's elastin stain and von Geesen counterstain to define the internal and external elastic laminae of the vessel wall. In cross section, muscular arteries had an intact circumferential muscular coat between the internal and external elastic laminae. Nonmuscular arteries had only a single elastic lamina and no muscle visible in their walls. Partially muscular arteries had some muscle visible in their walls, but did not contain a complete circumferential muscular layer.

Statistical Analysis

Statistical analysis was performed using analysis of variance (ANOVA) or contingency table analysis where appropriate. The significance of pairwise comparisons within each group was determined by post hoc testing with the Scheffe-f test at the 95% confidence limit. *P* values of less than .05 were considered significant.

RESULTS

Gross and Histological Results

In all animals with DH, spleen, stomach, and small bowel were present in the left chest and the lungs were markedly reduced in size. In all animals with DH and simultaneous TL, 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).

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

Macroscopic Vascular Analysis (> 100 μm in External Diameter)

In DH cases, whole lung arteriograms and arteriograms of lung slices showed a qualitative decrease in the overall size of the pulmonary vascular bed when compared with those of C cases. In DH/TL cases, the size of the pulmonary vascular bed was increased over that of both DH and C cases (Fig 3).

Quantitative computer digital analysis of arteriogram lung slices showed that total vessel area was lower in DH than in control cases. Conversely, DH/TL lungs had a three-fold increase in total large vessel area over that of DH lungs ($P = .003$). However, the ratio of large vessel area per unit lung area was similar in all groups (P = NS) (Table 1).

Microscopic Vascular Analysis (5 to 50 μm in External Diameter)

Microscopic morphometry of the capillary bed showed that in DH/TL lungs the total number of capillaries (tot cap #) was increased 24 times over that of DH and 4 times over that of C lungs ($P = .0001$). Again, however, because alveolar growth was also enhanced, the number of capillaries per alveolus (cap/alv) was similar in all groups (P = NS). In contrast, the number of capillaries per cm^2 lung

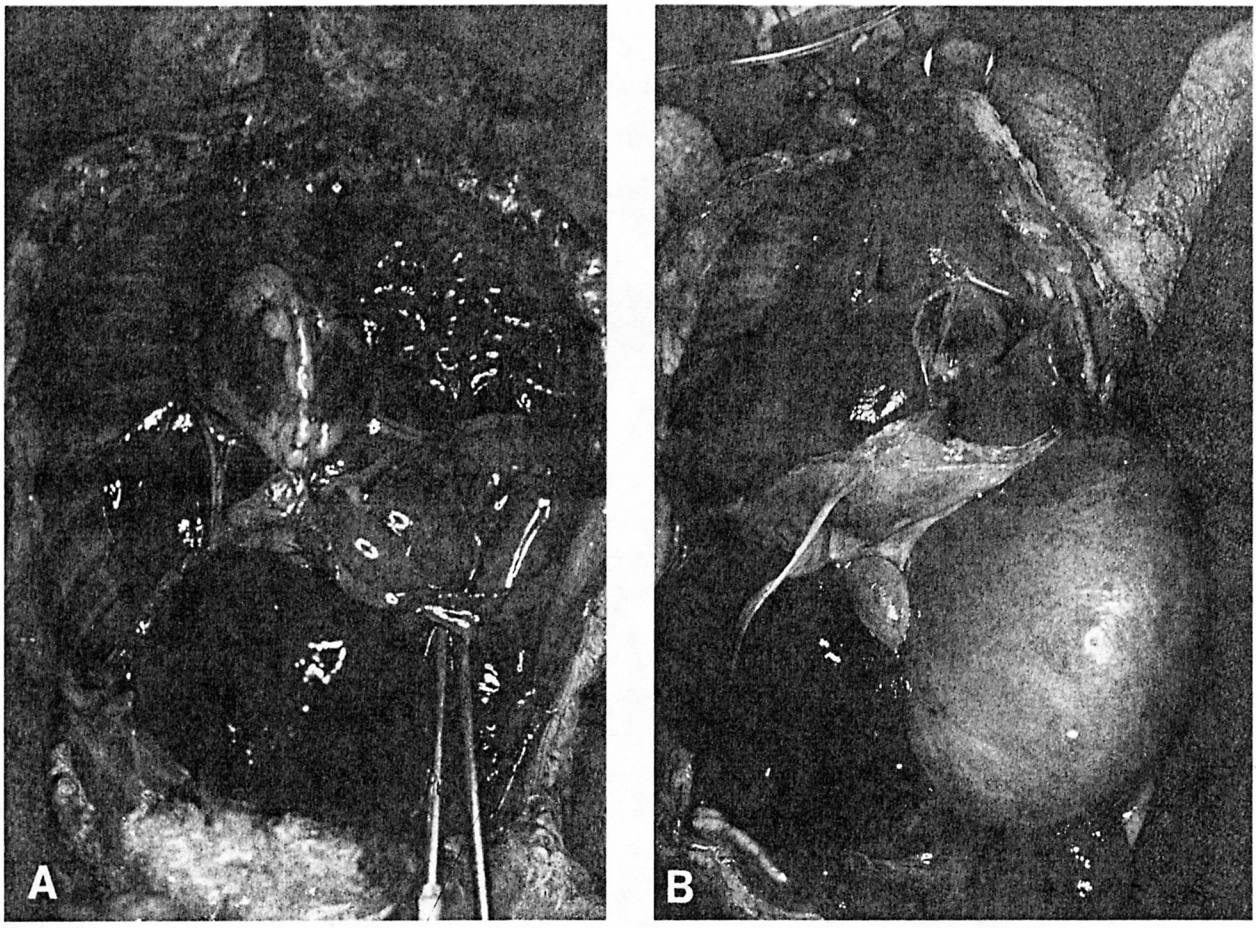

Fig 1. (A) DH animal, chest open via median sternotomy. Scissor passes through left diaphragmatic defect. Abdominal contents are present in left side of chest; lungs are small and not visible. (B) DH/TL animal, chest open via median sternotomy. The enlarged lung has completely reduced the herniated viscera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

tissue (cap/cm^2) was decreased in DH lungs by 50% when compared with either DH/TL or C lungs, which did not differ significantly from one another ($P = .0001$) (Table 1). In DH lungs, the apparent contradiction between a normal number of capillaries per alveolus and a decreased number of capillaries per cm^2 is explained by the decreased number of alveoli per cm^2 found in our previous morphometric analysis.[2]

Electron Microscopy

In DH/TL lungs, electron microscopy demonstrated normal capillary wall ultrastructure and normal thickness of the capillary-alveolar interface. In DH lungs, capillary ultrastructure was abnormal and the capillary-alveolar interface was thickened (Figs 4 through 6).

Arterial Muscularization

DH animals had three times as many fully muscularized vessels as DH/TL (46% versus 14%) and twice as many fully muscularized vessels as C animals (46% versus 19%) ($P = .0001$). Furthermore, fully muscularized vessels less than 100 μm in diameter were found only in the DH group. In fact, in DH lungs, 29% of vessels less than 100 μm in diameter were fully muscularized, whereas in DH/TL and C lungs, there were no fully muscularized vessels less than 100 μm in diameter ($P = .0009$). The smallest muscular vessels in DH/TL and C lungs were 224 and 117 μm respectively. In contrast, a full muscular coat was visible in the DH group in vessels as small as 17 μm. The percentage of nonmuscular vessels was increased in both DH/TL (64%) and C (54%) over DH lungs (16%) ($P = .0001$).

DISCUSSION

In fetal lamb models of DH using intrathoracic balloon inflation to simulate herniated viscera, Harrison et al produced fatal pulmonary hypoplasia and qualitative "reduction in the pulmonary vascular bed."[6] Simulated "correction of the hernia" by bal-

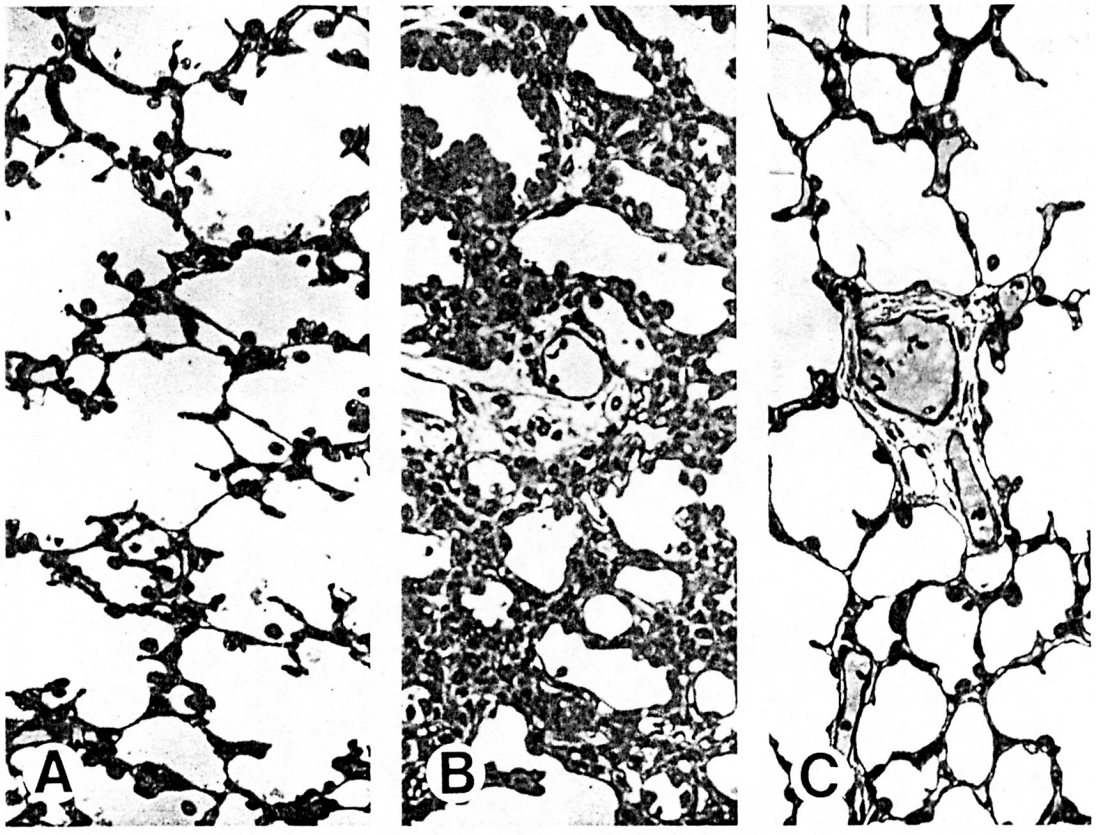

Fig 2. (A) Normal fetal lamb lung at 135 days' gestation. Note the thin alveolar septa and minimal amount of interstitial tissue. (Toluidine blue stained, plastic section, original magnification ×400.) (B) DH lamb lung at 135 days' gestation. When compared with Fig 2A, alveolar walls are markedly thickened, interstitial tissue is markedly increased, and alveolar air space is markedly diminished. (Toluidine blue stained, plastic section, original magnification ×400.) (C) DH/TL lamb lung at 135 days' gestation. Alveolar septa, interstitial tissue, and alveolar airspace have returned to normal. (Toluidine blue stained, plastic section, original magnification ×400.)

loon deflation allowed sufficient lung growth to achieve 100% survival as well as an apparent, but not statistically significant, increase in vascular volume by point-counting techniques.[7]

In a subsequent model in which DH was surgically created, Adzick et al reported a decrease in the total size of the pulmonary vascular bed, a decrease in the number of vessels per unit area of lung, and increased muscularization of the arterial tree.[8] Although statistical analysis was not performed, fetal surgical repair of DH appeared, qualitatively, to restore the pulmonary arterial bed toward normal.

We have previously shown that experimental fetal tracheal ligation accelerates fetal lung growth beyond even normal levels, reverses the alveolar pulmonary hypoplasia seen in surgically created fetal DH, and restores physiological function of the DH lungs to normal.[2] The present study was undertaken to determine whether the accelerated alveolar growth seen with fetal tracheal ligation in DH is accompanied by a parallel acceleration in growth of the macroscopic and microscopic pulmonary vasculature. Our data show six principle findings: (1) Experimental fetal DH results in hypoplasia of both large pulmonary vessels and the capillary bed. (2) Fetal tracheal ligation is capable of reversing these effects by accelerating both large vessel and capillary growth based on total large vessel area and total capillary number. (3) With TL, large vessel growth remains proportional to overall lung growth based on normal values for large vessel area per unit of lung area, and capillary growth remains proportional to alveolar growth based on normal values for cap/alv and cap/cm^2 lung tissue. (4) Experimental fetal DH results in increased muscularization of pulmonary arterial vessels as evidenced by an increased overall percentage of muscular arteries and by extension of muscle into normally nonmuscular vessels. (5) Fetal tracheal ligation reverses the increased muscularization of pulmonary arteries seen in experimental fetal DH. (6) By electron microscopy, capillary wall ultrastructure and the capillar-alveolar interface are abnormal in DH lungs and normal in DH/TL lungs.

Comparison of this fetal lamb DH model with

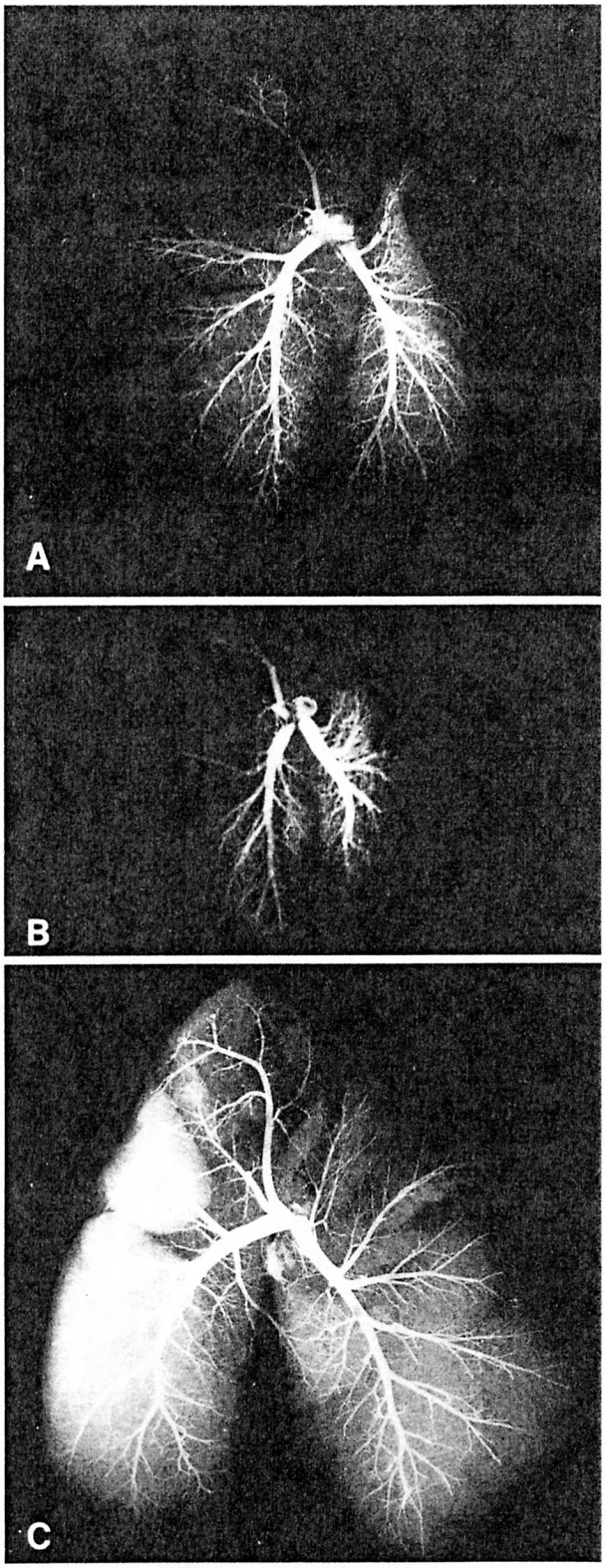

Fig 3. Pulmonary arteriograms of fetal lamb lungs at delivery. (A) Normal control, (B) DH lung, (C) DH/TL lung. Note the decrease in the overall size of the pulmonary vascular bed when DH lung is compared with control lung. In DH/TL lung, the size of the pulmonary vascular bed is increased over both DH and control lungs.

Table 1. Pulmonary Vascular Morphometrics

Parameter	C	DH	DH/TL	*P* Value
Total large vessel area ($\times 10^4$ pixels*)	1.48 ± .09	0.52 ± .13	1.50 ± .11	.003
Large vessel area/unit lung area	0.25 ± .01	0.25 ± .02	0.25 ± .02	NS
Tot cap # $\times 10^9$	2.7 ± 0.4	0.45 ± 0.1	10.8 ± 1.3	.0001
Cap/alv	1.02 ± .07	0.89 ± .12	1.06 ± .06	NS
Cap/cm^2 $\times 10^4$	9.1 ± 0.4	4.1 ± 0.5	9.3 ± 0.4	.0001

NOTE. Values are expressed as mean ± standard error of the mean.
*Pixel is a computer unit of area.

human infants dying of CDH indicates striking similarities in the pattern of pulmonary vascular abnormalities. In human CDH, Hislop and Reid documented a reduction in the total number of large "preacinar" vessels, but a stable ratio of these vessels per unit area of lung tissue.[9] In the intra-acinar region, the number of capillaries per square centimeter was decreased in CDH cases, while the number of capillarites per alveolus was unchanged, suggesting that the number of arteries in each acinus was

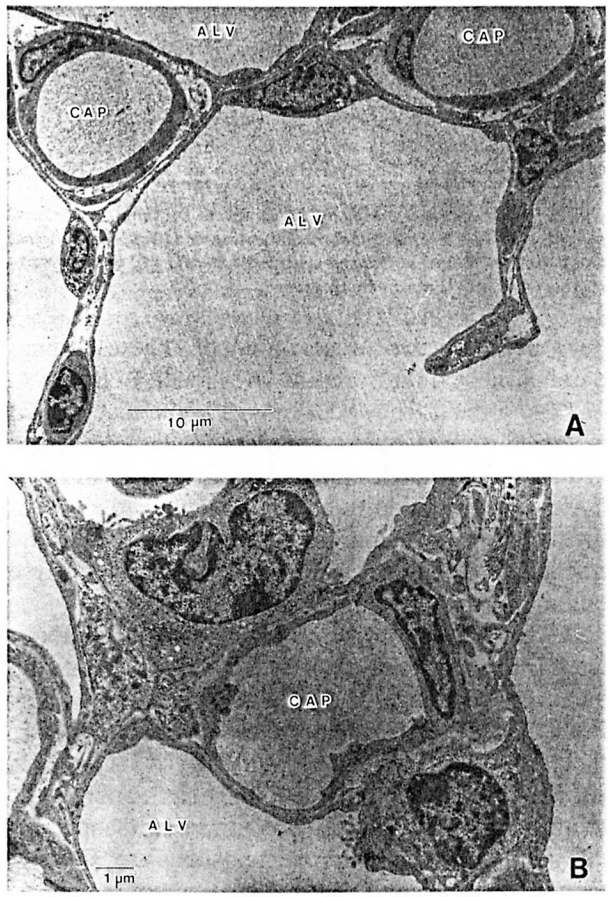

Fig 4. Transmission electron micrograph of normal fetal lamb lung at 135 days' gestation at (A) 3,400× original magnification and (B) 9,500× original magnification. Note the thin capillary-alveolar interface. CAP, capillary; ALV, alveolus.

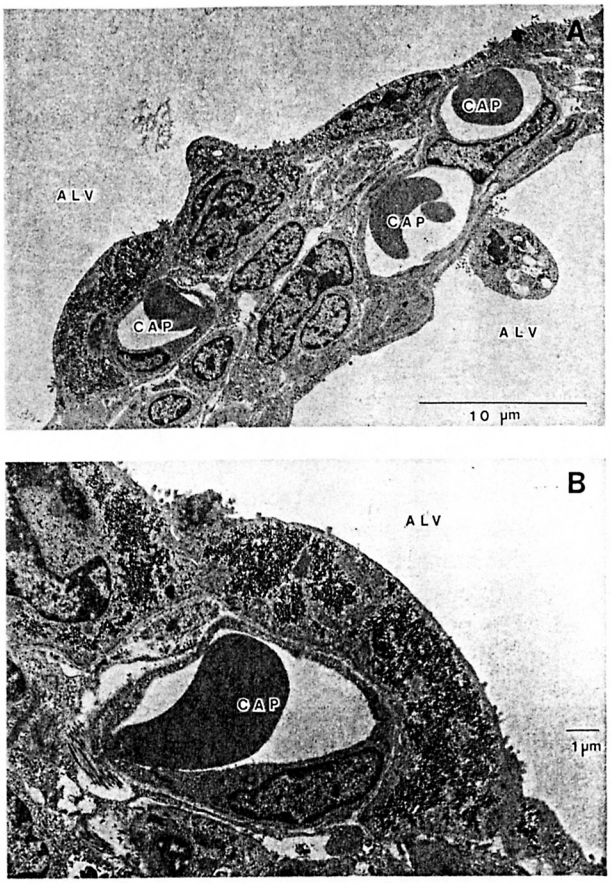

Fig 5. Transmission electron micrograph of DH lung at 135 days' gestation at (A) 4,500× original magnification and (B) 9,300× original magnification. When compared with Figs 4A and 4B, the capillary-alveolar interface is markedly thickened.

appropriate to the alveolar number. These findings parallel those of our present DH model. In addition, our DH animals showed a change in the distribution of small arterial vessels to more muscular vessels and extension of muscle into smaller arteries than is normal. These findings are again similar to those of investigations of human CDH.[9-12] In our model, fetal tracheal ligation was able to reverse all of these pulmonary vascular abnormalities.

We have previously shown that mechanically ventilated DH animals have poor gas exchange reaching a mean high of Pa_{O_2} of 51 mm Hg and a mean low Pa_{CO_2} of 144 mm Hg. DH/TL animals had improved function, achieving a mean high Pa_{O_2} of 360 mm Hg and a mean low Pa_{CO_2} of 42 mm Hg.[2] The results reported in the present study confirm our assumption that the significant improvements in gas exchange seen in animals with DH and TL could not have been possible without some pulmonary arterial growth to coincide with the acceleration of alveolar growth. The fact that pulmonary vascular growth in DH/TL appears so completely proportional to alveolar growth further supports our hypothesis that tracheal ligation accelerates pulmonary arterial and alveolar growth by enhancing mechanisms responsible for normal pulmonary development.

Previous electron microscopic studies of experimental DH by Pringle et al have shown a decrease in the complexity of the capillary network at the level of the alveolus.[13] Electron microscopy in the present study shows that DH is also associated with abnormal thickening of the capillary-alveolar interface and that this effect is also reversible by tracheal ligation.

In the normal human lung, thinning of the muscular walls of pulmonary arteries takes place rapidly after birth, being nearly complete by the age of 4 months.[14] We have previously shown that the increased arterial muscularization seen with human CDH also decreases with age, albeit slowly.[15] In the present study, DH/TL animals actually have a higher percentage of nonmuscular arteries than controls do, suggesting that fetal tracheal ligation may also accelerate a maturation process that normally occurs postnatally.

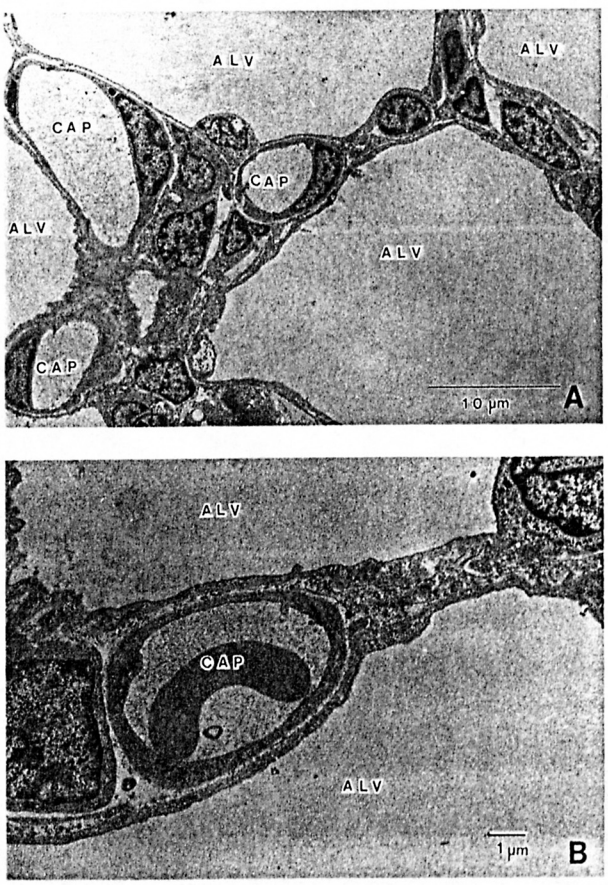

Fig 6. Transmission electron micrograph of DH/TL lung at 135 days' gestation at (A) 3,500× original magnification and (B) 9,800× original magnification. When compared with Figs 5A and 5B, the capillary-alveolar interface has returned to normal.

The functional implications of the structural changes presented here may be significant. Death in CDH is principally caused by either alveolar hypoplasia or unremitting pulmonary hypertension, presumably secondary to decreased size of the vascular bed and/or precocious muscularization. It is therefore encouraging that tracheal ligation seems to alleviate the detrimental effects of CDH on both alveolar growth and the vascular tree. Furthermore, the rapidity of the vascular changes seen in this study and the alveolar changes seen in our previous study[2] are well within the present time limits of ECMO support. What remains to be determined is whether these changes can only be achieved in the prenatal period, thereby necessitating consideration of fetal manipulation, or whether, with ECMO support, similar changes can be induced postnatally. Critical to this undertaking is the identification of the specific mechanisms (stretch, growth factors, etc) responsible for these phenomena. These investigations are currently ongoing in our laboratory.

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Discussion

S. Adzick (San Francisco, CA): I enjoyed your talk and I want to emphasize to the group here the high quality of work that you are doing. You are to be complimented.

The hallmark for the newborn with severe CDH is hypoplasia of the lung parenchyma as well as abnormalities of the pulmonary vascular tree. We've learned in fetal sheep with CDH that if you impede the normal egress of lung fluid by tracheal ligation, one gets astounding lung growth. These lungs reduce the viscera from the chest into the abdomen, and, importantly, have improved pulmonary function after birth.

What Dr DiFiore and his colleagues have shown us is that the pulmonary vascular abnormalities that correlate with the terrible clinical problem of pulmonary hypertension can be prevented by plugging the trachea before birth. I have one question and one comment.

Because you performed tracheal ligation at the same time you created the diaphragmatic defect, it might be more accurate to say that the pulmonary vascular changes are prevented as opposed to reversed by the ligation. My own bias is that established pulmonary hypoplasia can be reversed by tracheal ligation based on some of the experimental work we have done.

The comment is that at UCSF we now have IRB approval to do temporary tracheal occlusion in human fetuses with large diaphragmatic defects and fetal liver in the chest, a group that cannot be treated with complete diaphragmatic hernia repair before birth. What we have learned so far is that the biology of the process in human fetuses appears to mimic that in lambs and that one gets astounding lung growth within 2 to 3 weeks before birth after ligation.

My hope is that this will represent a new approach to what has been a frustrating clinical problem for all of us.

J.W. DiFiore (response): Your comment on the use of the term "reversal" is a good one. Our use of that descriptive term is based more on the previous tracheal ligation study, in which there were two groups. In the first group, we studied alveolar growth and the diaphragmatic hernia was created simultaneously with tracheal ligation as in this study.

In the second of half of that study, created a diaphragmatic hernia at 90 days' gestation and then performed tracheal ligation 20 days later at 110 days' gestation, and showed an improvement in function. That's really the origin of the conclusion that the defects in diaphragmatic hernia are actually reversed by tracheal ligation.