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Abolition of Fetal Breathing Movements by Spinal Cord Transection Leads to Reductions in Fetal Lung Liquid Volume, Lung Growth, and IGF-II Gene Expression

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ABSTRACT. Fetal breathing movements (FBM) are considered necessary for normal growth and structural maturation of the fetal lung, but the underlying mechanisms are unclear. The small fluctuations in lung dimensions caused by FBM have been proposed as a stimulus to lung growth, but it is equally possible that FBM act by maintaining the basal level of lung luminal volume, which is an established determinant of fetal lung growth. Our aim, therefore, was to determine the effects of abolishing FBM, while retaining the integrity of the diaphragm, on the volume and rate of production of fetal lung liquid, gene expression for IGF-II, and fetal lung growth. FBM were abolished in seven fetal sheep by high spinal cord transection at 114 ± 1.2 d of gestation; seven intact fetuses served as controls. At 119 to 124, 125 to 130, and 131 to 136 d, we measured the volume and secretion rate of lung liquid by dye dilution. At these three age ranges, the lungs of cord-transected fetuses contained 27 to 53% less lung liquid than controls (p = 0.004), and their rates of secretion were 65 to 138% greater (p = 0.001). At postmortem (135 ± 0.1 d), the lungs of the cord transected fetuses contained less DNA per kg body weight and tended to be lighter and to contain less protein than controls. IGF-II gene expression in the lungs of cord-transected fetuses was significantly less than that in controls. We conclude that the abolition of FBM causes an initial reduction in the degree of lung expansion, which eventually leads to lung hypoplasia, possibly mediated by reduced IGF-II gene expression. FBM probably contribute to the maintenance of fetal lung liquid volume, and hence lung growth, by opposing the loss of lung liquid caused by the elastic properties of the lungs. (*Pediatr Res* 34: 148–153, 1993)

Abbreviations

FBM, fetal breathing movement
SSC, sodium chloride sodium citrate solution

Studies in fetal animals and observations of human fetuses have indicated that FBM are an important stimulus for growth and structural maturation of the fetal lung. In humans, the absence of FBM as a result of phrenic nerve agenesis (1) or congenital abnormalities of the CNS (2) results in fetal lung hypoplasia. Experimental abolition of FBM by section of the phrenic nerves in fetal sheep (3–5) also results in hypoplasia and structural immaturity of the lungs. Because prolonged denervation of the diaphragm leads to atrophy of the diaphragm muscle, the associated lung hypoplasia could have resulted from upward displacement of the diaphragm and a reduction in thoracic volume. Thus, experiments were subsequently performed in fetal rabbits (6) and sheep (7) in which FBM were abolished, while the integrity of the phrenic nerves and diaphragm were retained. In these studies, the upper cervical spinal cord was transected, thereby avoiding diaphragmatic atrophy but preventing the rhythmic activation of phrenic motoneurons from brainstem respiratory neurons. These experiments (6, 7) resulted in pulmonary hypoplasia in the absence of diaphragmatic atrophy, leading to the suggestion (8–10) that the small fluctuations in thoracic and lung dimensions that are caused by FBM (11, 12) provide a necessary stimulus for normal lung growth and maturation.

It has been known for many years that the degree of fetal lung distension by its luminal liquid is an important determinant of lung growth and structural maturation. Prolonged obstruction of the fetal trachea, leading to the accumulation of pulmonary luminal liquid and overdistension of the lungs (13), results in pulmonary hyperplasia in rabbits (14) and sheep (15, 16); studies on the lungs of human infants with congenital laryngeal atresias have provided similar results (17, 18). In contrast, the prolonged removal of pulmonary liquid, preventing normal distension of the lungs, leads to pulmonary hypoplasia (15, 16). In view of the sensitivity of the developing lung to its level of distension, we considered it possible that the reduced lung growth that follows the abolition of FBM could be caused by a prolonged change in thoracic, and therefore pulmonary, volume rather than by the absence of phasic thoracic and pulmonary movements.

The aims of the present study were to determine the effects of the abolition of FBM by high cervical-cord transection on fetal lung liquid volume and production and to compare these with alterations in indices of fetal lung growth at the end of a treatment period. In essence, we have followed the experimental procedure adopted by Liggins *et al.* (7), but have monitored the volume of lung liquid and its rate of production and have terminated the experiments at an earlier fetal age. We have recently demonstrated in fetal sheep that reduced pulmonary growth produced by a sustained reduction in lung distension was associated with a reduction in IGF-II gene expression in pulmonary tissue, whereas overdistension of the lungs led to pulmonary hyperplasia and increased IGF-II gene expression (19, 20). Thus, a secondary aim was to determine whether reduced lung growth induced by high spinal-cord transection was associated with a decrease in IGF-II gene expression in fetal lung tissue as predicted by our earlier experiments.

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MATERIALS AND METHODS

Experiments were carried out using 14 pregnant Merino cross-bred ewes. Aseptic surgery on the ewe and fetus was performed, using halothane anesthesia, 107 to 118 (113.0 ± 0.8 SEM) d after mating. Two groups of fetuses were prepared. In seven fetuses, the spinal cord was transected between the first and second cervical vertebrae, whereas in the other group (controls, $n = 7$), the spinal cord remained intact. In all fetuses, catheters were implanted into a carotid artery and jugular vein (for blood sampling) and a pair of wide-diameter, re-entrant cannulas (for the measurement of lung liquid volume and production) were implanted into the cervical trachea (21, 22). A catheter was implanted into the amniotic sac for the measurement of amniotic sac pressure. After antibiotics (Depomycin 2 mL intramuscularly; Intervet, Lane Cove, Australia) were administered to the fetus, the uterine and abdominal incisions were closed. The ewes were housed in individual cages and allowed at least 5 d of recovery before any measurements were made.

Using an established indicator dilution technique (21, 23–25), we measured the volume and rate of production of fetal lung liquid between 119 and 124 d, 125 to 130 d and 131 to 136 d of gestation. During this procedure, the two halves of the tracheal loop were separated, and an impermeant dye (Dextran Blue 2000, Pharmacia, North Ryde, Australia) was added to the lung liquid via the descending cannula and its rate of dilution measured over 2 h. At all other times, the tracheal loop was intact, allowing the normal flow of tracheal fluid. Fetal blood samples were taken before each study to confirm fetal well-being.

At 134 to 136 d of gestation (mean 135.0 ± 0.1), the ewes and fetuses were painlessly killed by an i.v. overdose of pentobarbitone. The interval between surgery and postmortem was 20.2 ± 1.3 d for cord-sectioned animals and 22.6 ± 1.0 d for controls. Each fetus was weighed, and its lungs, diaphragm, and other major organs removed and weighed. Tissue samples were snap-frozen in liquid nitrogen and stored at −70°C for RNA, DNA, and protein analysis. Samples of lung tissue were dried for the estimation of dry lung weight. We confirmed that the cervical spinal cord had been sectioned in the treated animals. Fetal body weights at the time of the experiments were estimated from the body weight at death (26).

DNA estimation. The DNA content of fetal tissues was determined using a fluorometric assay (20, 27). Four samples of lung tissue from each fetus were used; they were taken from upper and lower lobes of left and right lungs. Tissue samples (0.5 g) were homogenized in cold phosphate buffer and centrifuged for 5 min at 2500 rpm. DNA standards (calf thymus DNA, Sigma Chemical Co., St. Louis, MO) were dissolved in phosphate buffer (pH 7.4) and diluted to give concentrations between 10 and 100 μg/mL. An EDTA solution and fluorochrome buffer (pH 7.4) were added to the standard solutions and to the diluted (1:5) supernatant from tissue samples. The standards and samples were incubated at room temperature in darkness before measuring their fluorescence at an excitation wavelength of 356 nm and an emission wavelength of 480 nm.

Protein estimation. Tissue protein contents were determined by dissolving tissue in 1 M NaOH at 90°C, followed by neutralization with HCl (1 M) before measuring the protein concentration using a standard protein assay (Bio-Rad, North Ryde, Australia). BSA standards were made up in distilled water and diluted to achieve concentrations of 100, 50, 25, 12.5, and 6.25 μg/mL.

Measurement of IGF-II mRNA levels. Relative IGF-II mRNA levels in fetal pulmonary tissue were determined by Northern blot analysis using a previously described ovine IGF-II probe and method (20, 28, 29). The analysis was performed on samples from the lower lobe of the left lung. Total RNA was prepared using a guanidine thiocyanate/cesium chloride method. The RNA (20 μg) was denatured, electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde, and then transferred to a nylon membrane (Zeta Probe, Bio-Rad Labs, Cambridge, MA). The blot was prehybridized for 3 to 4 h and then hybridized with a ^{32}P-labeled ovine IGF-II cDNA probe ($1\ 2 \times 10^6$ CPM/mL) at 42°C for 16 to 18 h. After hybridization, the blots were washed twice in 1× SSC (0.1% SDS) and once in 0.1× SSC (0.1% SDS) for 30 min at 42°C before being air-dried and subjected to autoradiography using intensifying screens at −70°C. To determine relative amounts of total RNA present on the membrane, the blot was stripped by washing in 0.1× SSC (0.5% SDS) at 90°C for 1 h and then reprobed with a radiolabeled cDNA for 18S ribosomal RNA (a gift of Dr. D. Denhardt, Rutgers University, NJ). The relative levels of IGF-II mRNA on the autoradiogram were quantified using laser densitometry (Ultrascan XL, LKB, Stockholm, Sweden) and were measured as arbitrary absorbance units. These values were subsequently corrected for minor loading differences between lanes by expressing the density of the IGF-II bands as a proportion of the density of the 18S ribosomal band for that lane.

Data analysis and presentation. Data were analyzed by analysis of variance and t test where appropriate. Results are presented as mean and SEM.

This project was approved by the Monash University Standing Committee for Ethics in Animal Experimentation.

RESULTS

As indicated by their blood gas status, all fetuses were considered to be in good condition throughout the studies. At the time of making the lung liquid volume and secretion rate measurements, blood gas values for the two groups were not significantly different. Mean values were: P_{O_2}, 3.1 ± 0.1 kPa (23.2 ± 0.8 mm Hg); P_{CO_2}, 5.7 ± 0.1 kPa (42.8 ± 0.9 mm Hg); pH, 7.368 ± 0.004; arterial saturation, 65.6 ± 1.4%. At postmortem, the body weights and wet weights of major organs of cord transected fetuses were not significantly different from those of control fetuses.

Episodes of tracheal pressure fluctuations (FBM) were present in all control fetuses. Recordings of tracheal pressure from cord-transected fetuses revealed episodes of pressure fluctuations resembling FBM, but the amplitudes were smaller than those in control fetuses. When pressure was recorded from the lower tracheal cannula alone (*i.e.* isolated from the upper trachea) in cord-transected fetuses, no pressure fluctuations were recorded, confirming that they were not of thoracic origin. When recordings were made from the upper tracheal cannula isolated from the lower catheter, episodes of pressure fluctuations resembling FBM were detected in all cord-transected fetuses (Fig. 1). As in control fetuses, the periods of tracheal pressure fluctuations in cord-transected fetuses were associated with increased efflux of tracheal fluid, as detected by the flowmeter (Fig. 1).

Lung liquid volumes were significantly lower ($p = 0.004$) in cord-transected fetuses than in controls (Table 1). The mean lung liquid volume measured in cord-transected fetuses at 119 to 124 d (9.0 ± 0.8 d after the abolition of FBM) was 46.7% of that measured in control fetuses at the same age range. Between 125 and 130 d, the mean lung liquid volume in cord-transected fetuses was 69.0% of that in control fetuses, and between 131 and 135 d, mean lung liquid volume in cord-transected fetuses was 72.7% of that in control fetuses. The mean reduction in lung liquid volume over the three age ranges was 37%. Figure 2 shows lung liquid volumes, adjusted for estimated body weight, in the two groups of fetuses.

Lung liquid production rates were significantly greater ($p = 0.001$) in cord-transected fetuses than in controls (Table 1, Fig. 3). Within the youngest age range, production rates in cord transected fetuses were 65.6% greater than those of controls. At 125 to 130 d and at 131 to 136 d, lung liquid production rates in the cord-transected fetuses were, respectively, 138 and 95% greater than in controls. The mean increase in secretion rate over the three age ranges was 99%.

Details of the pulmonary weights, protein and DNA contents

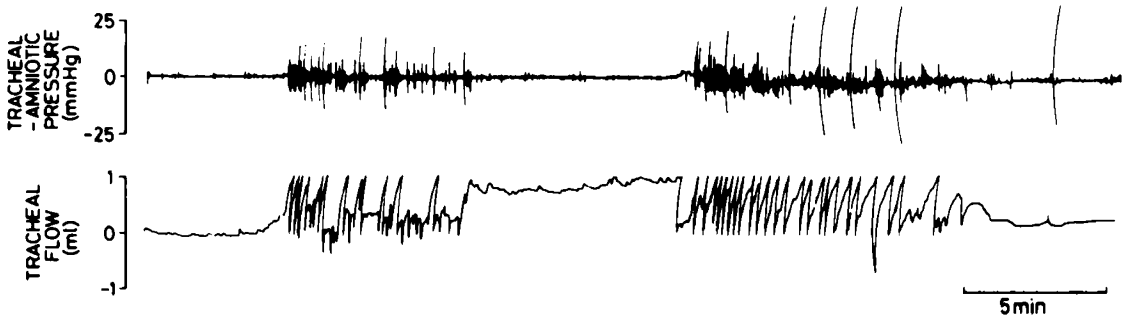

Fig. 1. Polygraph recording from a fetal sheep after spinal cord transection. Recordings are of tracheal pressure (minus amniotic sac pressure) and integrated tracheal fluid flow. Two episodes of tracheal pressure fluctuations, thought to be generated by rhythmic activation of laryngeal dilator muscles, coincide with increased efflux of liquid from the lungs. Flowmeter integrator resets with each 1 mL of tracheal fluid efflux or influx (*upward deflection* indicates efflux).

Table 1. *Ages at which studies were performed on six spinal cord-transected (cord-x) and six control fetuses, their lung liquid volumes, and lung liquid production rates*

	Age ranges (d)		
	119–124	125–130	131–136
Fetal age (d)			
Cord-x	121.8 ± 0.7	128.0 ± 0.2	133.4 ± 0.5
Control	120.8 ± 0.5	126.3 ± 0.7	133.0 ± 0.3
Lung liquid volume (mL)			
Cord-x	31.5 ± 5.1	54.7 ± 4.1	77.9 ± 6.4
Control	67.6 ± 7.5	79.1 ± 10.9	107.1 ± 7.0
Lung liquid production (mL/h)			
Cord-x	8.3 ± 0.7	15.1 ± 1.3	17.7 ± 1.0
Control	5.0 ± 0.9	6.4 ± 1.2	9.1 ± 1.6

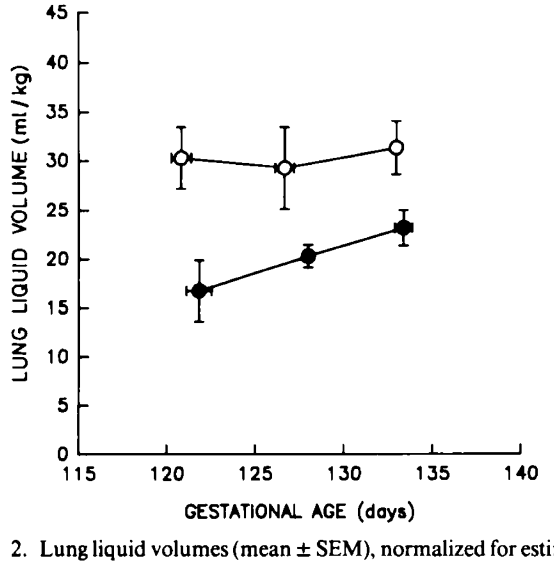

Fig. 2. Lung liquid volumes (mean ± SEM), normalized for estimated body weight, in control fetuses and fetuses with transected spinal cords.

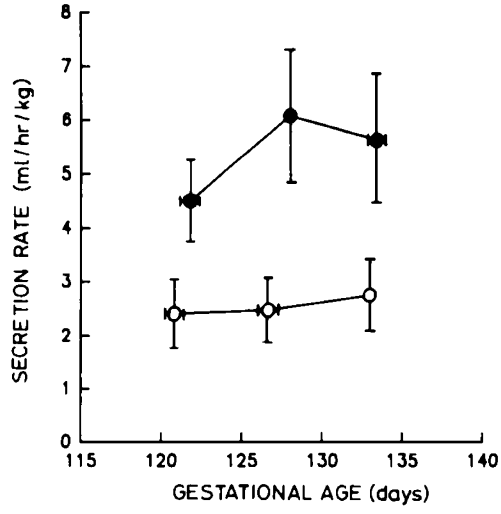

Fig. 3. Rates of lung liquid production (mean ± SEM), normalized for estimated body weight, in control fetuses and in fetuses with transected spinal cords.

Table 2. *Body weights, pulmonary weights, and DNA and protein contents in control and cord-transected fetuses*

	Controls	Spinal cord—transected
Body weight (kg)	3.68 ± 0.2 (n = 7)	3.76 ± 0.4 (n = 7)
Wet lung wt (g/kg body wt)	27.9 ± 1.0 (n = 7)	24.3 ± 1.4 (n = 7)
Dry lung wt (g/kg body wt)	3.00 ± 0.27 (n = 7)	2.27 ± 0.13 (n = 7)
Lung DNA concentration (mg DNA/g wet lung wt)	2.41 ± 0.12 (n = 7)	2.11 ± 0.11 (n = 7)
Lung DNA content (mg DNA/kg body wt)	67.1 ± 3.4 (n = 7)	50.9 ± 3.2 (n = 7)
Lung protein content (mg/kg body wt)	355.0 ± 27.9 (n = 7)	288.1 ± 29.6 (n = 7)

in the two groups of fetuses are given in Table 2. Wet and dry lung weights, relative to body weight, tended to be less in cord-transected fetuses than in controls. The DNA content of the lungs, relative to body weights, in cord-transected fetuses was 24% less ($p = 0.003$) than in controls; the concentration of DNA in the lungs was not different between the groups. The mean total protein content of the lungs, relative to body weight, from cord-transected fetuses was 19% lower than that of control fetuses, but this difference was not statistically significant due to wide variations between animals.

At least six IGF-II transcripts (6.0 to 1.2 kb) were detected in pulmonary tissue from both control and cord-transected fetuses (Fig. 4). When all IGF-II mRNA species were combined, total IGF-II mRNA levels in lung tissue were found to be significantly higher ($p < 0.05$) in control fetuses compared with cord-transected fetuses (4.5 ± 0.5 *versus* 1.9 ± 0.4 IGF-II/18S ratio; Figs. 4 and 5).

There were no significant differences in the wet or dry weights of the diaphragm relative to body weight between the two groups. Nor were there significant differences between the DNA concentration or content in the diaphragm tissue from the two groups.

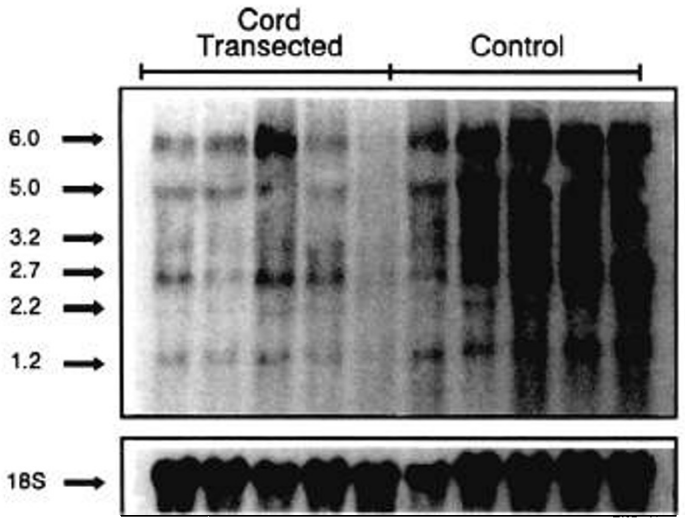

Fig. 4. Northern blot hybridization of total RNA (20 μg/lane) from lungs (lower left lobe) of spinal cord-transected fetuses (*lanes 1–5*) and control fetuses (*lanes 6–10*) probed with ^{32}P-labeled ovine IGF-II cDNA (*top panel*) and with ^{32}P-labeled 18S ribosomal RNA cDNA. This Northern blot was exposed on XAR film (Kodak) for 8 h at −70°C. The relative densities of IGF-II mRNA bands were expressed as a proportion of the density of the 18S RNA band to compensate for minor loading differences between lanes.

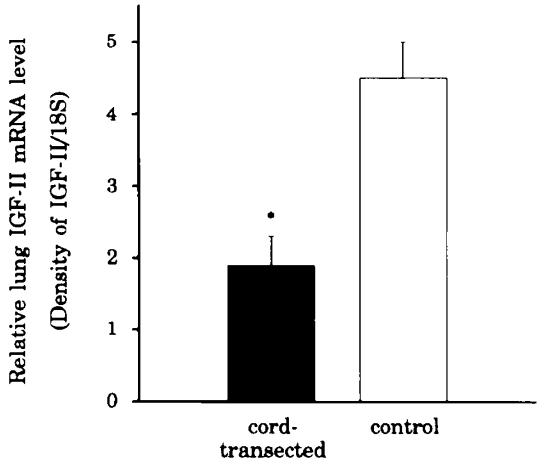

Fig. 5. Relative IGF-II mRNA levels in fetal lung tissue (*lower left lobe*) collected from control fetuses (*white bar*) and spinal cord-transected fetuses (*black bar*). IGF-II mRNA levels are presented as relative absorption units (cm^2) and are expressed as a proportion of the density of the 18S RNA band for each lane to account for minor differences in loading between lanes. *Asterisk* indicates a value that is significantly different from control value.

DISCUSSION

This experiment has shown that the abolition of FBM, in the absence of diaphragmatic atrophy, leads to a sustained reduction in the volume of lung liquid. Within 7 to 12 d of abolishing FBM, lung liquid volume had declined by approximately 50%. In accordance with observations in fetal rabbits (6) and fetal sheep (7), the lungs of our treated animals showed evidence of being hypoplastic, as indicated by their lower DNA content, and tendency for lower dry weight and protein content. In relation to their weight, the lungs of cord-transected fetuses at postmortem contained an amount of lung liquid similar to that of control fetuses. However, in relation to body weight, the volume of liquid was substantially reduced. We consider it most likely that the fetal lung hypoplasia detected in our cord-transected fetuses was due to the prolonged and substantial reduction in lung liquid volume. The cause of the reduced size of the lungs of treated fetuses cannot be identified with certainty, but is most likely due to an initial reduction in lung expansion (*i.e.* reduced luminal volume relative to lung size) resulting from the abolition of FBM. This would be expected to cause a reduction in lung tissue growth such that, by the end of the 20-d study period, the lungs were hypoplastic and, compared with controls, contained less luminal liquid relative to body weight but normal amounts of liquid relative to lung tissue weight. A reduction in lung liquid volume and lung size at postmortem has been reported in two previous studies in which FBM were abolished (3, 4). In those studies, however, the reduced lung liquid volume has been attributed to atrophy of the diaphragm muscle after phrenic nerve section. In our experiments, the reduced lung liquid volume cannot have been due to diaphragmatic atrophy.

It is interesting that the reduction in total lung DNA content in spinal cord-transected fetuses (24%) is similar to the reduction (27.1%) in lung liquid volume after 20 d in the absence of FBM. The reductions in lung weights and DNA contents in our cord-transected fetuses were not as large as those found in a similar study by Liggins *et al.* (7); however, the mean treatment period in that study was 10 d greater than ours. It is well established that a prolonged reduction in fetal lung liquid volume will lead to hypoplastic changes in the lungs. Fetal lung hypoplasia results from prolonged reductions in lung liquid volume that are caused by diaphragmatic herniation (30), tracheal fluid drainage (15, 16, 20), pleural effusions (31), and oligohydramnios (24). In fetal sheep, prolonged oligohydramnios during late gestation caused a reduction in lung liquid volume of 22% and a reduction in dry lung weight, relative to body weight, of 18% (24).

The cause of the reduction in lung liquid volume after high cervical spinal transection in our experiments is not obvious, but it cannot be attributed to a reduction in lung liquid production, as this was found to be increased. It seems most likely that the reduced volume was due to the abolition of the thoracic component of FBM in the presence of continued phasic activity in the muscles of the upper respiratory tract. The presence of intermittent tracheal pressure fluctuations, which were recorded only in the upper trachea, and periods of augmented tracheal fluid efflux after spinal cord transection can be attributed to the normal FBM-related activity of the laryngeal dilator muscles (32). Rhythmic laryngeal dilation associated with FBM is capable of phasically lowering tracheal pressure (33) and is the likely cause of tracheal pressure fluctuations after bilateral phrenic nerve section (34).

In the fetus, the lungs develop in a distended state, and there is evidence that this distension is actively maintained against a tendency for the lungs to partially collapse due to their elastic recoil properties. During periods of apnea, the elastic recoil is opposed by narrowing of the glottis (32) and an increased resistance of the upper airway (35), which result in a reduced efflux of lung liquid and a supraamniotic pressure in the trachea (36). During episodes of FBM, rhythmic laryngeal dilation and an absence of active laryngeal constriction (32) result in a reduced upper-airway resistance (35), enabling the pulmonary elastic recoil to cause liquid to flow from the lungs at an increased rate (22). We propose that the diaphragmatic component of FBM has the effect of retarding the efflux of liquid from the lungs in the presence of laryngeal dilation, thereby helping to maintain lung liquid volume and the degree of lung distension.

After spinal cord transection, the escape of lung liquid during periods of apnea is still opposed by constriction of the larynx, as the innervation of the laryngeal muscles is not affected by spinal section. During episodes of central breathing activity that persist after high cord section, rhythmic laryngeal dilation still occurs, as evidenced by our recordings of periods of pressure fluctuations in the upper trachea and the presence of augmented tracheal fluid efflux during these periods. Under these circumstances, passive recoil of the lung is relatively unopposed, due to diaphragmatic quiescence, and larger than normal volumes of fluid are

lost from the lungs. This increased efflux need only be quite small over a prolonged period to cause a substantial reduction in lung liquid volume and would be difficult to detect in recordings of tracheal flow.

It is not clear why the production of lung liquid greatly increased as a result of spinal cord transection. Although we did not measure circulating hormone concentrations in these studies, we know of no hormonal mechanism that could account for such a large increase in lung liquid production. It is unlikely that disturbances of circulating catecholamines or vasopressin, both of which inhibit lung liquid production (23, 25, 37), occurred after spinal cord transection. It is possible that transection of the spinal cord resulted in an increase in pulmonary blood flow as a result of removing sympathetic drive to the pulmonary resistance vessels. A reduction in pulmonary blood flow has been shown to reduce the production of lung liquid (38), although we know of no evidence that an increase in pulmonary blood flow in the fetus will result in increased lung liquid production. It is also possible that a small reduction in luminal pressure that would be expected to occur in the presence of a reduced lung luminal volume could favor the production of lung liquid. In this regard, it is of interest that Fewell *et al.* (4) found that, after the abolition of FBM by phrenic nerve section, lung liquid production was unchanged, but relative to estimated lung weight, it may have increased.

The findings of this study are in agreement with our recent observation that up to seven IGF-II mRNA transcripts are expressed in fetal lungs, with the 6.0- and 5.0-kb mRNA being the most abundant (20, 39). It is likely that the genomic organization of the ovine IGF-II gene is similar to that of humans, which contains nine exons and four different promoters (40). The presence of multiple IGF-II transcript sizes is, therefore, most probably due to alternate RNA splicing, differential promoter use and differential polyadenylation (40). It is not known whether all IGF-II mRNA transcripts produced by the fetal lung are translated into the mature peptide, although in humans all transcripts, except for the smallest (1.8 kb), are known to contain coding regions for the mature IGF-II peptide (40). Potentially, therefore, these mRNA transcripts can be translated to form precursor IGF-II proteins, although the translational potential could vary considerably between transcripts.

Our findings of a prolonged reduction in lung liquid volume after spinal cord transection of the fetus and a subsequent reduction in lung growth in association with a significant reduction in relative levels of pulmonary IGF-II mRNA are consistent with our previous findings that alterations in pulmonary growth caused by sustained alterations in lung liquid volume in fetal sheep are associated with corresponding changes in IGF-II gene expression in pulmonary tissue (19, 20). This study, therefore, provides further evidence that an alteration in IGF-II gene expression may be causally involved in mediating the effect of altered lung distension on fetal lung growth. It has been shown that, in fetal lungs, IGF-II mRNA are localized in fibroblasts of the mesenchyme and interlobular septa, which form much of the structural matrix of the developing lung (29, 41). Thus, the IGF-II gene is expressed in tissue structures and cell types ideally suited to sense changes in lung expansion. The IGF-II produced by these cells could, in turn, exert its mitogenic activity by autocrine or paracrine mechanisms. Numerous other growth factors are also produced by the fetal lung, including growth-promoting and growth-inhibiting factors, any of which may also be involved in mediating the effect of distension on lung growth.

In light of our findings, it may be necessary to reconsider the conclusions from previous studies in which FBM have been either abolished by spinal cord transection (6, 7) or attenuated by increasing the compliance of the thoracic walls (42). Our data lead us to the conclusion that the stimulatory effects of fetal breathing on fetal lung development are principally mediated by their action in maintaining lung liquid volume, although the rhythmic stresses imposed on lung tissue (10) may also play a role. Furthermore, it now seems likely that the abolition of fetal breathing, as well as diaphragmatic atrophy, accounted for the fetal lung hypoplasia that followed section of the phrenic nerves (3, 4).

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