Spontaneous Peristaltic Airway Contractions Propel Lung Liquid through the Bronchial Tree of Intact and Fetal Lung Explants

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Spontaneous contractions of the fetal airways are a well recognized but poorly characterized phenomenon. In the present study spontaneous narrowing of the airways was analyzed in freshly isolated lungs from early to late gestation in fetal pigs and rabbits and in cultured fetal mouse lungs. Propagating waves of contraction traveling proximal to distal were observed in fresh lungs throughout gestation which displaced the lung liquid along the lumen. In the pseudoglandular and canalicular stages (fetal pigs) the frequency ranged from 2.3 to 3.3 contractions/min with a 39 to 46% maximum reduction of lumen diameter. In the saccular stage (rabbit) the frequency was 10 to 12/min with a narrowing of $\sim$ 30%. In the organ cultures the waves of narrowing started at the trachea in whole lungs, or at the main bronchus in lobes (5.2 $\pm$ 1.5 contractions/min, 22 $\pm$ 8% reduction of lumen diameter), and as they proceeded distally along the epithelial tubes the luminal liquid was shifted toward the terminal tubules, which expanded the endbuds. As the tubules relaxed the flow of liquid was reversed. Thus the behavior of airway smooth muscle in the fetal lung is phasic in type (like gastrointestinal muscle) in contrast to that in postnatal lung, where it is tonic. An intraluminal positive pressure of 2.33 $\pm$ 0.77 cm H_2O was recorded in rabbit fetal trachea. It is proposed that the active tone of the smooth muscle maintains the positive intraluminal pressure and acts as a stimulus to lung growth via the force exerted across the airway wall and adjacent parenchyma. The expansion of the compliant endbuds by the fluid shifts at the airway tip may promote their growth into the surrounding mesenchyme.

Spontaneous narrowing and relaxation of the airways in the developing fetal lung has been described since the early part of the twentieth century. These spontaneous contractions are characteristic of phasic smooth muscle where regular bursts of action potentials give rise to rhythmic mechanical activity, typified by the smooth muscle of the viscera (1). Yet the contraction of mammalian airway smooth muscle in postnatal life is characterized by slow, graded contractions leading to airway narrowing and occurs without the generation of action potentials during membrane depolarization (2, 3). This is classified as tonic smooth muscle, in common with many blood vessels (1). Perhaps the rhythmic contractile activity of fetal airway smooth muscle is not so surprising since the intestine and the lung have a common embryological origin, with the lung developing as an outgrowth of the foregut in the late embryonic stage (4). Nevertheless, it indicates that airway smooth muscle would need to lose its capacity to generate spontaneous electrical activity sometime during fetal life or at birth and acquire the characteristics of tonic smooth muscle. If and when this occurs is unknown.

The phenomenon of spontaneous narrowing of airways in the fetal lung was first observed in explants of lung in culture from embryos of chicken (5) and guinea pigs (6). More recently it was reported in explants of first-trimester human lung (7) and in gestation Day 11 mouse lung (8), where by 48 h in culture, spontaneous contractions began. However, much less information is available for intact fetal airways. McCray (7) noted spontaneous activity of epithelial tubules in small fragments of fresh lungs from first-trimester human fetuses and characterized their contractile responses to pharmacologic agents (discussed later). Sparrow and colleagues (9, 10) video-recorded sequences of strong spontaneous narrowing of individual airways in the isolated intact bronchial tree in the late first and early second trimesters of fetal pig lung. The spontaneous narrowing moved the lung liquid backward and forward along the airway lumen. The airways also contracted strongly to many agonists, e.g., acetylcholine (ACh), histamine, and Substance P, displaying a force per cross-sectional area of smooth muscle and a sensitivity comparable with that of the airways of postnatal animals (9–11). They relax with β-agonists in explants (7) and throughout the airways of the freshly isolated fetal bronchial tree (9, 10). The spontaneous activity is unaffected by tetrodoxin, indicating that neural activity is not essential, and by atropine, showing that endogenous ACh is not involved. In addition, this rhythmic mechanical activity ceases in the presence of calcium antagonists—indicative of action potentials carried by an inward Ca^{2+} current via voltage-operated channels in intact airways (9) and in culture (7). Thus, it can be concluded that these spontaneous contractions are of myogenic origin. But why the airway smooth muscle displays mechanical activity so early in gestation and whether it has functional significance *in vivo* remain unknown.

During gestation, lung liquid is secreted by the epithelial cells into the lumen of the future airways against a closed glottis (12). Preventing drainage by occluding the trachea causes hyperplasia and a concurrent increase in insulin-like growth factor (IGF)-II in the lungs of fetal lambs during late gestation. The opposite, draining off the lung liquid, causes hypoplasia and a reduction in IGF-II (13). Recently Blewett and associates (14) have simulated this approach in explants of fetal mouse lung, where occlusion of the lobar bronchus produces a larger, more mature lung lobe with an intraluminal pressure of 3.1 mm Hg compared with 0.8 mm Hg in an unligated one. It is likely that airway smooth-muscle tone may well be producing these pressures in what otherwise would be a very compliant epithelial tubule.

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Abbreviations: acetylcholine, ACh; inner diameter, i.d.; insulin-like growth factor, IGF.

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In the present work we have sought to quantify spontaneous activity—extent of narrowing and relaxation, rhythm, characteristics of propagated spontaneous narrowing, and the pressure of the lung liquid and its displacement during pulsatile activity—in intact airways and in explants of fetal mouse lung. To see whether spontaneous narrowing is related to the stages of lung development, we carried this out from the early pseudoglandular phase to the saccular phase using the pig fetal lung in the first and second trimesters and in fetal rabbits in the late third trimester.

Materials and Methods

Fetal Pigs

A total of 17 fetal pigs were collected from 11 pregnant sows (Large White/Landrace hybrids, freshly slaughtered at a local abattoir) 20 to 30 min after death, packed on ice, and transported back to the laboratory, where the body weights and crown–rump lengths were measured. The lungs were cut off at the midtrachea, removed, and placed in cold, gassed Krebs solution (9). Fetuses were chosen weighing between 6 and 10 g (32 to 36 d gestation, early pseudoglandular stage, six fetuses), 25 to 35 g (44 to 48 d gestation, late pseudoglandular stage, six fetuses), and 90 to 120 g (56 to 60 d gestation, canalicular stage, five fetuses) according to the published data of Pomeroy (15), as well as Altman and Dittmer (16). Gestation in the pig is 116 d (body weight at birth: $\sim$ 1,100 g). These gestation times may be overestimates, given that growth rates of modern hybrid pigs are much faster than traditional species. With a dissecting microscope the parenchyma and vasculature were carefully teased from the bronchial tree, which was kept immersed in cold Krebs. In lungs from the early and late pseudoglandular stage the whole bronchial tree was used, but above a body weight of 30 g it was divided at the lobar bronchi. It was then placed in a water-jacketed glass bath coated on the bottom with Silgard 184 silicone elastomer (Dow Corning, Midland, MI) to form a chamber of 5 ml volume through which a steady flow of Krebs (5 to 10 ml/min) gassed with 95% O_2/5% CO_2 at 32°C was perfused. The bronchial tree was kept in place with steel pins of 100 μm diameter placed through the proximal trachea and distal ends of the stem bronchi. A video image of the bronchial tree was obtained with a color video camera (Sony DXC 151 AP) attached to a dissecting microscope by using a suitable intensity of transmitted light and displayed on a color video monitor. The edges of the airway lumen were defined using transmitted light (9, 10) with suitable side lighting added from a cold-light, fiberoptic source as required. A Super VHS video recording of the contracting airways was obtained by scanning along the airways of the bronchial tree at low power and zooming in on a site where spontaneous contractions were occurring (Sony color video camera DXC-151AP; Panasonic video recorder NV-FS90 HQ). The three-dimensional nature of the bronchial tree, the tendency of the smaller airways to float in the physiologic saline, the limited distance along any one airway where the transmitted light clearly revealed the lumen, and the movement of the contracting airway made the focusing difficult at the magnification required. Thus, it was rarely possible to capture a complete sequence of spontaneous activity from onset to cessation. The trachea and proximal stem bronchi were generally not suitable for imaging because of the opacity of the cartilage in the wall (9, 10).

Nomenclature of the Airways and Assessment of Lung-Development Stages in Fetal Pigs

The bronchial tree of the pig comprises two stem bronchi each with five laterals (L) designated according to their sequence of development (17). They were numbered L1 to L5 on each bronchus, with L1 arising from the trachea on the right-hand side. The structures of the bronchial trees of 8- and 20-g fetal pig lungs have been depicted earlier (9, 10), where the diameters of the stem bronchi, laterals and their secondary and tertiary branches are given. The stages of lung development in both the pig and the rabbit were determined from hematoxylin and eosin–stained paraffin sections, 5 μm thick, according to the criteria of Burri and Moschopulos (18).

Video Imaging Analysis of Fetal Pig Lungs

To quantify narrowing, a sequence of frames of a contracting region was captured from the videotape at 1- or 2-s intervals onto a Macintosh Power PC 8500. In general, two or three adjacent sites lying in the laterals and/or their subsequent branches were selected where the images of the lumen were sharp, and a length of lumen $\sim$ 600 μm uninterrupted by large branch points was available. The internal diameter (i.d.) was quantified using NIH-Image 1.61 software (NIH, Bethesda, MD) by drawing a line with the cursor across the airway wall perpendicular to the epithelium. These sites were identifiable during narrowing and relaxation because of morphologic features, particulate matter trapped in the wall, or minute artifacts introduced into the outer wall during dissection of the bronchial tree. At least three measurements were made at each site for airways $<$ 300 μm and six for $<$ 100 μm and a mean was taken (coefficient of variation for the latter was 6%). At the maximum magnification used, 1 pixel = 4 μm.

The recordings of spontaneous activity shown in the figures are typical of the other lungs taken at approximately the same developmental stage. The spontaneous activity in airways of the bronchial tree was a complex, variable, and transient phenomenon that commenced after an indeterminate period of quiescence, and exhibited a series of pulsatile contractions before diminishing in strength and resuming quiescence. Thus, the data for the mechanical activity (duration, velocity) are shown as the mean and range for the particular fetal lung used.

Fetal Rabbits

Lungs from six fetuses were taken from three pregnant rabbits (New Zealand White) in the third trimester ($\sim$ 27 d gestation, saccular stage, body weight 25 $\pm$ 3.3 g, term 33 d). The doe was killed by cervical dislocation, and the fetuses by decapitation and exsanguination. The parenchyma and pulmonary arterial bed were removed, and the bronchial tree was placed in a deeper chamber modified from that described earlier so as to maintain the temperature of the preparation at 37°C. The video-imaging analysis was performed in the same way as for the fetal pig lungs. The studies using fetal pigs and rabbits were approved by the Animal Ethics Committee of the University of Western Australia.

Lung Intraluminal and Interstitial Pressure

Pregnant rabbits were lightly anesthetized, and fetuses were delivered by caesarian section with the umbilical cord still connected. One fetus at a time was removed and placed on a thin, flat, stainless-steel plate lying on the surface of the mother's abdomen. For the measurement of the intraluminal pressure the trachea was carefully exposed from the ventral aspect with as little disturbance as possible to its attachments to the adjacent tissue. In one movement the trachea was penetrated by a sharp stainless-steel needle, gauge #30, which was attached to a Statham Gould low-pressure transducer via a liquid-filled system. The pressure change from zero cm H_2O was recorded on a moving chart during impalement for a minimum of 5 min. Immediately after recording, an intraperitoneal injection of a neuromuscular blocking agent was administered to relax the diaphragm, in preparation for static compliance measurements. If lung liquid was seen

to leak from the site of entry during the impalement the measurement was discarded.

The interstitial pressure was determined by micropuncture of the lung. The micropuncture was performed after opening a "pleural window" (19, 20) through resection of the superficial tissues and intercostal muscles under stereomicroscopic view using fine surgical tools. This allowed exposure of a portion of lung surface about 2 mm^2. The animal was then placed on a heated pad in lateral decubitus with the pleural window up and was covered with a plastic wrap to avoid dehydration. The animal was paralyzed by intraperitoneally injecting Pancuronium bromide (0.2 mg/kg body weight). For micropuncture we used glass micropipettes whose tips had been beveled down to a diameter of 2 to 3 μm, with a taper length of 200 μm. Micropipettes were filled with 0.5 M NaCl solution, colored with lissamine green (for a better visibility of the micropipettes), and previously filtered through 0.2-μm Millipore filters. Micropipettes were calibrated in the range ± 30 cm H_2O. They were then placed in a holder connected to a Gould P23XL pressure transducer motor driven by a servonulling system (Instrumentation for Physiology and Medicine, Model 5A). An electrical zero was obtained before and after each measurement by inserting the pipette into a small saline pool grounded to the animal and placed at the same height of the micropuncture site. Micropuncture was performed by advancing the pipette under stereomicroscopic view (×60) at an angle of about 45 degrees relative to the surface of the lung. A site within the pleural window was chosen where the liquid-filled sacculi were clearly detected as black dots of irregular size surrounded by well-defined translucent mesenchymal tissue stripes 50 to 100 μm wide. Recording depths within the lung tissue ranged from 50 to 150 μm. Criteria for accepting micropipettes recordings were: (*1*) a stable recording, (*2*) an unchanged electrical zero on withdrawing the pipette, and (*3*) similar values (within ± 2 cm H_2O) on at least three consecutive attempts. We obtained reliable data from three animals.

These experiments were carried out in the laboratory of Professor Giuseppe Miserocchi, Department of Physiology, University of Milan, Milan, Italy, with the approval of the Animal Ethics Committee of the University of Milan.

Lung Organ Culture

Fetal mouse lungs were obtained from 11- to 15.5-d-old embryos under sterile conditions and cultured according to Schuger and coworkers (21). They were removed after cutting through the proximal trachea. The microdissection was done in phosphate-buffered saline (127 mM NaCl, and 10 mM Na_2HPO_4, pH 7.4). The lungs were cultured at the air–culture medium interface on a floating filter (TSTP Isopore filter, 13-mm diameter, 3-μm pore size; Millipore, Bedford, MA) in a 24-well plate (Falcon-Becton Dickinson, Lincoln Park, NJ) at 37°C in 5% CO_2/air. Dulbecco's modified Eagle's medium (GIBCO BRL-Life Technologies, Basel, Switzerland), was enriched with 10% fetal calf serum (GIBCO BRL), 5 μg/ml bovine insulin (Sigma Chemical Co., St. Louis, MO), 2 mM glutamine (Sigma), 75 μg/ml streptomycin (Sigma), and 100 μg/ml penicillin (Sigma). Three lung explants were transferred onto each filter and placed in a well containing 350 μl medium. The distances between the three explants were large enough to ensure that the tissues did not touch during growth. The explants were fed daily by adding 200 μl of fresh medium per well. For gestation Days 11 to 12, whole lungs were used. For Days 12.5 to 15.5, the five lobes of the lungs were separated by cutting the main bronchi and cultured individually. The explants were observed using an inverted microscope (Diaphot-TMD; Nikon, Tokyo, Japan) and the movements were recorded on a digital video recorder (DHR-1000VC; Sony, Tokyo, Japan) using a color video camera (Optronics LE-470; Visitron Systems, Puchheim, Germany). After 10 min the plates were moved back to the incubator for rewarming to 37°C. For measuring the i.d. of the airways, images were captured at a rate of one frame/second on a Pentium-based computer system containing a capture board (DVBK-1000E; Sony) and printed in black and white at 100- to 200-fold magnification for the mouse-lung explants and in color at 166-fold magnification for the rabbit lungs. The studies using fetal mouse lungs were approved and supervised by the Animal Ethics Committee of the Canton of Bern.

All cultured mouse-lung explants (more than 300 from ~ 40 pregnant mice) exhibited spontaneous contractions, but not all explants were used for this study. A total of 18 whole lungs from six pregnant mice were randomly chosen at gestation Days 11 ± 0.5 (pseudoglandular stage = embryonic Days 9.5 to 16.6) (22). At Days 12.5, 13.5, 14.5, and 15.5 the five lobes of the lungs were separated and cultured individually. The lobes of interest were randomly chosen from at least six lungs of two pregnant mice for every embryonic day. The explants were studied after 2 to 4 d in culture. The frequencies of the contractions were determined by counting the contractions over time, either directly in the microscope or on the videotape.

Results

Fetal Rabbit Lung

The lungs of fetal rabbits in the third trimester (27 d gestation, body weight 25 ± 3.3 g) were in the saccular stage, i.e., the acini comprise terminal clusters of widened air spaces called saccules. After equilibrating in Krebs for 30 min at 37°C, the airways showed sporadic spontaneous activity localized to a few regions at any one time. In general, rhythmic contractions developed quickly in an airway and lasted from 30 to 90 s before weakening in amplitude and becoming quiescent. Resumption of activity was very unpredictable. Small-bore airways (~ 100 μm i.d.) tended to resume contracting again after 1 to 2 min, but large-bore airways (~ 450 μm i.d.) did so at infrequent intervals. Figure 1 shows a video image of a 1,400-μm section of a lateral airway in which mechanical activity occurred along its length (Figure 1, *upper panel*) and recorded during the period when mechanical activity was strongest. At the three sites shown, the extent of narrowing was determined from images captured at 1-s intervals (Figure 1, *middle panel*). The contractions averaged 10/min (range 10 to 12), but were not strictly consistent in shape or duration. For example, the duration of the fourth and fifth contractions at site 1 are shorter (3.5 s each) compared with the average duration of 5.2 s (range 4.5–6 s) for the other 17 contractions shown. This appears to be because the fourth contraction has aborted after 1 s, relaxes briefly, then resumes contracting. Irregular contractions were common events. The delay in the arrival of the narrowing wave from site 1 to site 2 and then to site 3 (denoted by the rhombus) was used when feasible to obtain an estimate of velocity of propagation along the wall (mean 29 μm/s, range 25 to 33). The bore of the lumen was narrowed by about one-third as the waves were conducted along the airway in a distal direction. This example is representative of not less than five similar recordings made in the proximal and distal airways of each fetal lung ($n = 6$).

The movement of the lung liquid in the lumen of the airway was observed by following the path of a cluster of cell debris suspended in the liquid. These clusters chiefly

comprise adhering cell nuclei that collect at intervals along the airways (9). Figure 1, *lower panel*, shows the displacement of the lung liquid with each contraction. Before time zero the airway was essentially quiescent and the cell debris stationary, lying close to site 2. The progressive relaxation of the airway wall from site 1 to site 3 in the first 5 s (Figure 1, *middle panel*) causes the liquid to move proximally in the sixth second and pause for 1 s before being propelled distally at 7 s. Thereafter, the pulsatile activity moves the liquid distally and proximally with each wave of narrowing and relaxation. The shorter, less powerful contractions 4 and 5 recorded at site 1 (Figure 1, *middle panel*) appear to affect the extent of lung liquid flow at 20 to 25 s.

This type of pulsatile activity was the most commonly encountered activity in the laterals and subsequent generations of airways from the lungs of the six fetal rabbit pups studied. However, in the stem bronchi ($>$ 300 μm lumen diameter) powerful propulsive responses were often observed. For example, a strong, propagated wave of contraction would develop rapidly, narrowing the i.d. from 324 to 204 μm in 1 s (37% narrowing). This wave was only slightly diminished in strength 381 μm distally, and at a further 454 μm still narrowed the internal diameter by 27% (219 to 161 μm in 1 s). The wave dissipated quickly with two small successive contractions in the next 7 s, leaving this segment of the airway still partially narrowed.

Fetal Pig Lung

Spontaneous narrowing was observed in proximal and distal airways of the bronchial tree of fetal pig lungs in the pseudoglandular (3 to 8 wk) and canalicular (7 to 13 wk) stages, a period which approximated to the first and second trimesters. The structure and nomenclature of the airways of the bronchial tree were described earlier (*see* MATERIALS AND METHODS). Spontaneous activity was observed intermittently throughout the bronchial tree (trachea excepted) at 32 to 33°C in small fetuses ($<$ 10 g, gestation age $<$ 5 wk) where the airway wall was sufficiently thin for the lumen to be imaged. As the fetuses enlarged, the walls of the more proximal stem bronchi became too opaque to visualize the lumen, so recording of narrowing was restricted to the laterals and their branches. At $>$ 11 wk gestation (317 g body weight) the adventitia covering the wall of the bronchial tree was too thick and opaque to visualize the lumen. In general, the spontaneous activity exhibited by the fetal pig airways was similar to that of the rabbit, where strong rhythmic contractions were propagated over a distance of several millimeters and did not persist for more than several minutes. Small, localized fluctuations in lumen diameter were also seen in the small-bore tubules ($<$ 100 μm i.d.), occurring within 100 to 200 μm of the terminal sacs.

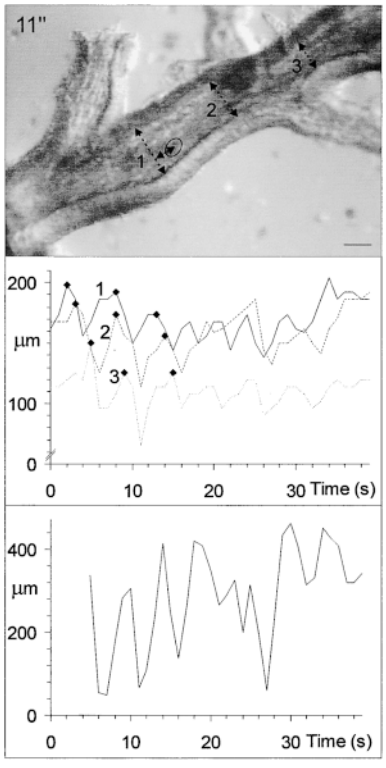

Figure 1. Fetal rabbit lung. (*Upper panel*) Video image of a length of an airway of the isolated bronchial tree of a fetal rabbit lung at Day 27 of gestation. The magnitude of the spontaneous contractions was measured at the three sites shown. *Bar* = 100 μm. (*Middle panel*) Spontaneous narrowing and relaxation plotted as lumen diameter (μm) against time at sites 1, 2, and 3. Sites 1 and 3 are 651 μm apart. The commencement of narrowing is indicated by a *rhombus* in the first three contractions. (*Lower panel*) The movement of the lung liquid in the lumen of the airway is plotted as the distance moved by a suspended cluster of cell debris (*upper panel, circle*) against time. Upward indicates movement in the distal direction. In the first 5 s the cell debris was stationary. For a video sequence of the *upper panel, see* http://www.ana.unibe.ch/media/SponCon/

Figure 2, *upper panel*, shows an example of spontaneous narrowing in a distal tubule ($\sim$ 90 μm i.d.) of a fetal pig lung (5 wk gestation, 9.7 g) that was in its early pseudoglandular stage. Here the tubule comprises a layer of epithelial cells surrounded by a layer of airway smooth muscle one or two cells thick, invested by a loose mesenchyme containing a nerve network (9, 10). The mechanical activity is plotted at two points 500 μm apart along a tubule that branched off the third lateral on the left side. The tubule narrowed and relaxed regularly for several minutes at a frequency of 2.3 contractions/min. However, the amplitude fluctuated, reaching $>$ 50% of maximum lumen diameter at times. In the section of the recording shown, the amplitude is 39% of the maximum lumen diameter at site 1 and 49% at site 2. The wave of contraction traveled from site 1 to site 2 at a mean velocity of 67 μm/s (range 62 to 71, four contractions).

Figure 2, *middle panel*, shows a series of regular phasic contractions recorded near the base of the second lateral (300 μm i.d.) later in the pseudoglandular stage (6.5 wk gestation age, 35 g body weight). As the wave of mechanical activity was propagated distally, the frequency of contractions (3.3/min) could be recognized in a small secondary branch ($\sim$ 100 μm i.d.) arising from this lateral at a distance of 150 μm and also at a further 335 μm distally. Although the contractions appear less regular, this is due partly to the difficulty in accurately pinpointing the precise position of these distal sites as the airway narrows and contracts, thereby increasing the variability of the lumen diameter determinations.

Figure 2, *lower panel*, shows a short sequence of spontaneous narrowing in the airways of a fetal pig lung in the canalicular stage (8.1 wk gestation, 95 g). The successive narrowing and relaxing cycles (frequency 2.3/min) were initially determined at a position along the fifth lateral where the lumen diameter was 235 μm (site 1), and thereafter in a distal direction at 308 μm (142 μm i.d.), and at a further 463 μm (99 μm i.d.). The narrowing phase occurred quickly (2 to 4 s) and was followed by a much slower relaxation (14 to 22 s). This narrowing rate was faster than in the

pseudoglandular stage (Figure 2, *middle panel*) and the relaxation rate was slower, presumably a reflection of the more mature airway wall with a greater smooth-muscle layer and a thicker wall. The amplitudes of narrowing at sites 1, 2, and 3 were 42, 43, and 46%, respectively.

The frequency of spontaneous narrowing ranged from 2.1 to 3.7/min in the fetuses ($n = 17$) used at these stages of lung development. The spontaneous narrowing of the airway wall moved the lung liquid contained within the lumen in a distal direction, and as the wall relaxed the flow of lung liquid was reversed as described for the rabbit lung.

Fetal Mouse Lung Organ Culture

Explants of fetal mouse lung were obtained at embryonic Days 11 to 15.5 and cultured at the air–culture medium interface. Spontaneous contractions were observed in the epithelial tubules within 48 h of culture in all cultured explants. In cultures of separate lobes (embryonic Days 12.5 to 15.5), the contractions originated proximally at the lobar bronchus and traveled to the periphery (Figure 3), whereas in complete lungs (embryonic Days 11 to 12) the narrowing started at the trachea (Figure 4). The frequency of spontaneous narrowing ranged from 2.8 to 7.8 contractions/min (mean = 5.2 ± 1.5, $n = 13$ lungs, taken at embryonic Day 12.5 and cultured for 3 d), but for a single explant the frequency did not fluctuate by more than 20% during the 10 min observation. Neither the embryonic days before culture nor the number of days in culture influenced the frequency per se.

During contractions the lobar bronchus shown in Figure 3, site 1, narrowed by 38 ± 4% ($n = 30$) (i.e., contractions) of its mean relaxed diameter, but the amplitude decreased at sites 2 and 3 to 27 ± 3% ($n = 30$) and 17 ± 12 ($n = 30$), respectively (Figure 3, *lower panel*). The airways in this particular lung contracted strongly compared with the mean of the seven lungs used (e.g., 22 ± 8% in the lobar bronchus).

By observing aggregates of cellular debris inside the lumen of the epithelial tubules, the flow of the lung liquid during the spontaneous narrowing could be followed (Figure 4). The wave of contraction produced a movement/shift of liquid toward the periphery, ending at the walls of the terminal buds, that was sufficient to move the cellular debris from the bifurcation more than halfway to a terminal endbud (Figure 4). Widening of one terminal endbud at the moment when the wave of moving liquid reached the end of the epithelial tubules is shown in Figure 4, *lower panel*. Upon relaxation, the lung liquid flowed back into the widening proximal regions of the tubules and the terminal endbuds narrowed again.

Intraluminal and Interstitial Pressure of Fetal Rabbit Lungs

Positive pressures were consistently recorded on penetrating the wall of the trachea of lungs from ~ 27-d-gestation fetal rabbits (late canalicular/saccular stage). The mean pressure was 2.33 ± 0.77 cm H_2O ($n = 7$ lungs). After the

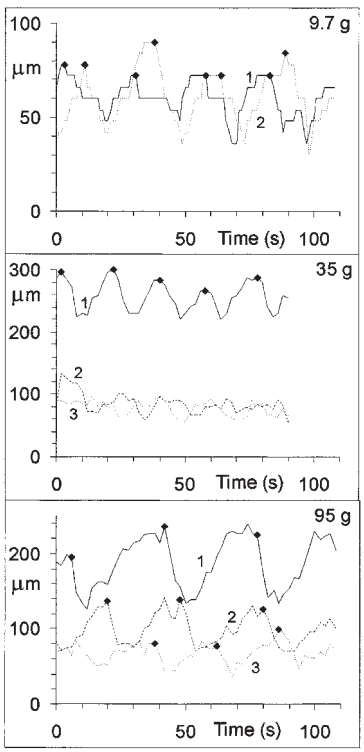

Figure 2. Fetal pig lung. Spontaneous narrowing in the airways of fetal pig lungs in the early- (*upper panel*) and mid- (*middle panel*) pseudoglandular stages, and the canalicular (*lower panel*) stage. (*Upper panel*) A sequence of spontaneous narrowing in the tubules of the lung from a fetal pig at 5 wk gestation (9.7 g body weight) are plotted as lumen diameter (µm) against time at two sites 500 µm apart. (*Middle panel*) Spontaneous contractions at 6.5 wk gestation (35 g body weight) were recorded in a lateral (site 1) and a secondary branch (sites 2 and 3) at distances of 150 and 485 µm distal from site 1. (*Lower panel*) A propagating wave of contraction was observed in a 95-g (8.1-wk gestation) fetal pig. Sites 2 and 3 are 308 and 771 µm more distal from site 1. In all three panels the commencement of narrowing is indicated by a *rhombus.*

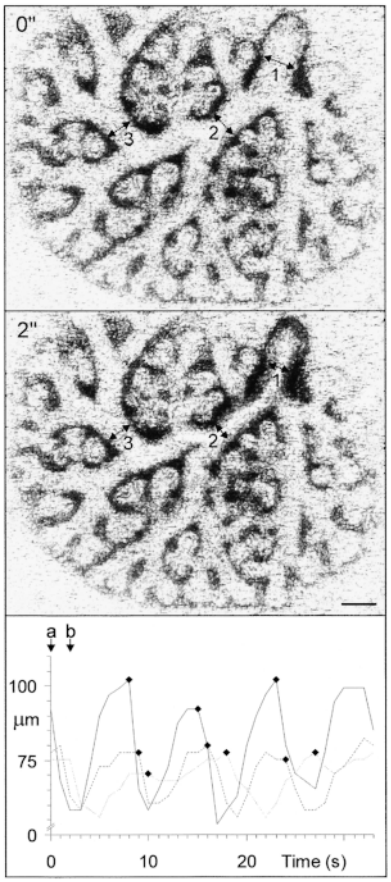

Figure 3. Fetal mouse lung in culture—spontaneous contractions. A right lower lobe at embryonic Day 12.5 was cultured for 3 d. The diameter of the epithelial tubules is measured at the lobar bronchus (point 1, *solid line* in *lower panel*), at point 2 (*dashed line* in *lower panel*), and more distally at point 3 (*dotted line* in *lower panel*). The *upper panel* shows the explant at the commencement of the narrowing (*arrow a* in *lower panel*) and the *middle panel* shows the same explant 2 s later, when the wave of contraction just reached point 2 (*arrow b* in *lower panel*). In the *lower panel* the lumen diameters are plotted versus time. The beginning of each contraction is marked with a *rhombus. Bar:* 100 µm. For a video sequence of this explant, *see* http://www.ana.unibe.ch/media/SponCon/

initial fluctuations in pressure due to deformation and movement of the trachea by the impalement had subsided, a steady-state pressure was maintained.

In terms of the biologic function of a pressure it may be more important to know the pressure gradient than the absolute pressure. Therefore, we measured the interstitial pressure surrounding the small airways. In fetal rabbit lungs from ~ 27 d after gestation the interstitial pressure averaged 0 ± 0.7 cm H_2O (n = 3). We concluded that the intraluminal pressure is equal to the transmural pressure (pressure gradient across the walls of the future airways).

Discussion

In the present study we have characterized some of the mechanical features of spontaneous narrowing in the future airways of the isolated bronchial trees of fetal pigs and rabbits and in the epithelial tubules of fetal mouse lung explants in culture. Spontaneous contractions were observed in both proximal and distal airways to within 100 μm of the terminal sac. They were not limited to the early fetal lung but occurred throughout the stages of lung development and persisting into the late third trimester. The lumen was narrowed by as much as 46% of its relaxed diameter, and the waves of narrowing were propagated distally over several generations of airways. In the freshly isolated bronchial trees the onset of rhythmic contractions was rapid, and they continued for more than one minute before weakening and disappearing to start again at a different part of the bronchial tree. The activities persisted for several hours after removal of the lung from the animal. Likewise, in lung explants, weak and infrequent spontaneous activities were observed after 24 h in culture. By 2 d, strong waves of narrowing were observed along the main lobar bronchi and laterals extending to the terminal sacs.

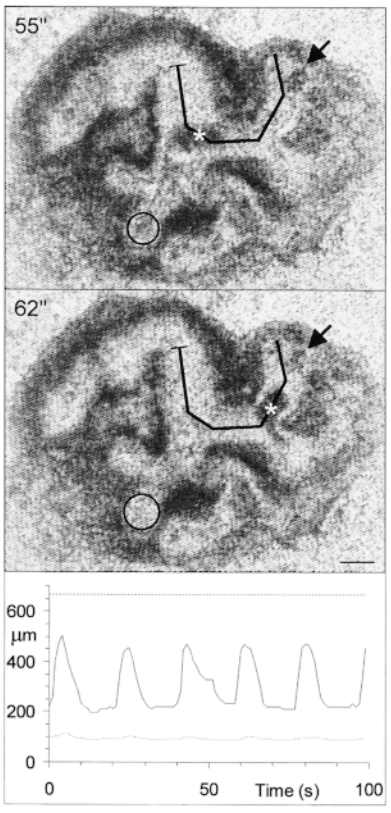

Figure 4. Fetal mouse lung in culture—movement of liquid. A complete fetal mouse lung at embryonic Day 11 was cultured for 3 d. The movement of the fluid inside the tubules was followed by the movement of aggregated cellular debris (*asterisks* in *upper* and *middle panels*). The *upper panel* shows a proximal and the *middle panel* a distal position of the particles (55 and 62 s after the start of the observation). The *lower panel* shows a plot of the displacement of the particles toward the periphery (*solid line*) and the synchronous alteration of the diameter of one of the terminal endbuds (*dotted line* in *lower panel* and *circles* in *upper* and *middle panels*). The *dashed line* in the *lower panel* indicates the end of the epithelial tubule. In the *upper* and *middle panels* the *solid lines* indicate the path measured; each starting point is the *bar* in the trachea. The *asterisk* indicates the center of the aggregated particles. The *arrows* point to a foreign body that moved toward the wall when the wave reached the terminal endbud. *Bar:* 100 μm. A video sequence of this figure is available at http://www.ana.unibe.ch/media/SponCon/

Their frequency varied from 4 to 7/min between lungs, which was not related to the days in culture. Roman (8) reported a frequency of 2/min but found that the frequency and strength varied between explants and were strongly temperature-dependent. We also observed the temperature dependency, but avoided a slowing of the frequency by controlling the temperature. The differences in the frequency of contractions of the fetal pig and rabbit lungs are most likely to be due to the temperatures used: fetal pig, ~ 2 to 3.3/min, 32 to 33°C; and fetal rabbit lung, 10 to 12/min, 37°C. These rhythmic, spontaneous contractions of the mouse lung in culture and of the freshly isolated bronchial tree are similar to those observed in isolated segments of postnatal gastrointestinal and uterine wall where the smooth muscle is classified as phasic. This contrasts with that of postnatal airways (and of most blood vessels) where the smooth muscle is stable, does not generate action potentials or contract spontaneously, and is classified as tonic (1). Thus, even in culture the airway smooth muscle still retains the characteristics of a phasic muscle, as was first observed by Lewis (5) in explants of chick embryo, and subsequently in lung explants of guinea pig, rat, human, and mouse (7, 8, 23, 24).

The narrowing was due to the airway smooth muscle that completely encircles the airways and lies perpendicular to their long axis. The musculature extends from the base of the terminal endbud, comprising a layer of newly differentiated cells, to the proximal trachea (10, 25, 26). It maintains this cylindrical arrangement around the airways from the early pseudoglandular stage to postnatal life, except in the trachea, where the formation of the cartilage rings later in the pseudoglandular stage reduces the muscle layer to that of a narrow segment on the dorsal side (26). This airway smooth muscle is functional from the terminal airways to the stem bronchi throughout fetal life, as our quantitative observations of spontaneous narrowing revealed.

The factors promoting spontaneous activity have not been examined in the current experiments. In general, spontaneous activity is initiated by stretching smooth muscle, by contractile agonists such as excitatory neurotransmitters (e.g., ACh), and by prostaglandins and other chemical mediators (for details, *see* the opening paragraphs of this article). The likelihood of the latter agents being endogenously present is unknown but would seem unlikely to occur both in fresh preparations and in culture. Temperature also affects spontaneous activity. As it falls from 37 to ~ 25°C, the frequency of the contractions slows (7) in parallel to the rate of force development, but the maximum force generated is generally unchanged or may increase (27). Functional cholinergic nerve fibers extend to the distal airways late in the first trimester in fetal pig

lungs (28), but spontaneous activity continues in the presence of atropine (10). It has been shown in the gastrointestinal tract that the spontaneous activity originates in the "Interstitial Cells of Cajal." These cells display pacemaker activity and are electrically connected to the longitudinal and circular smooth-muscle cells by gap junctions, thereby inducing the basal electrical rhythm (29). Their presence in the fetal lung is unknown. However, in other smooth-muscle tissues rhythmic activity appears to be generated by groups of smooth-muscle cells well connected by gap junctions to each other and to the bulk of the smooth muscle (30). This would appear to be the more likely situation in our lung preparations *in vitro* and in culture.

A striking feature was the pulsatile movement of the lung liquid in the lumen of the airways of the isolated bronchial tree and also in the lung explants. This was readily seen by the movement of cell debris carried in the moving lung liquid. Sometimes the debris was aggregated, but more often it was finely dispersed throughout the liquid (9). The liquid was driven distally in almost all cases by the distal-traveling peristaltic wave of contraction and aided by the increasing compliance of the thin-walled terminal tubules and their epithelial buds (9). If there was a hole or break in the airway wall, the liquid was driven toward the hole with some fluid partially exiting as a viscous drop, which did not mix with the surrounding Krebs. As the airway wall relaxed it withdrew back into the lumen (9). The minute pressure transients driving the liquid distally might be predicted to produce a transient increase in the volume of the terminal sacs. Because in the isolated bronchial trees only a few terminal regions were preserved well enough to be studied, expansion of the buds was an infrequent observation. However, it was readily observed during spontaneous contractions in our cultured lung explants, and McCray (7) and Roman (8) also noted this phenomenon in mouse lung explants.

The direction of the movement of the liquid at the onset of a spontaneous contraction can be thus envisaged. At the first moment of the contraction the liquid is pushed equally in both directions, but as the wave of contraction begins to move distally more of the liquid will follow it rather than move proximally (where it has to pass through the more contracted part of the tubule). The walls of the terminal endbuds comprise a single layer of undifferentiated epithelial cells, so they are likely to be more compliant than the airways and able to accommodate the minute volume of liquid moved. Upon relaxation of the airway wall after the spontaneous contraction, the liquid moves back, and the terminal endbuds return to their normal volume. For most of the contractions, the time between each contraction was long enough to allow a complete flow-back.

The lung liquid is under pressure in the airways of the bronchial tree of the fetal lung. In late-gestation, fetal rabbit lungs we measured an intraluminal pressure of 2.33 cm H_2O in the trachea and an interstitial pressure of 0 cm H_2O. Therefore, we conclude that the transmural pressure (the pressure across the wall) is equal to the intraluminal pressure. The recorded intraluminal pressure could be an underestimate because pressure loss on impaling the delicate tracheal wall was a potential source of error if liquid escaped before the ligatures were secured. Indirect estimates have been made in the late-term fetal lamb of ~ 2 mm Hg (31) and 1 to 2.8 mm Hg (32) using an indwelling tracheal cannula. After closure the pressure builds up, and this maintained pressure stimulates IGF-II production and lung growth, leading to a hyperplastic lung. An open cannula that drains off the liquid leads to a hypoplastic lung (13). In cultured mouse lung, Blewett and associates (14) have measured a pressure of 3.1 mm Hg in the lobar bronchus 3 d after ligation, compared with 0.8 mm Hg in the unligated lobe. As in the sheep experiments, ligation of the lung explants led to hyperplasia and to an acceleration of lung development *in vitro*. Similar results were observed for the rescue of oligohydraneous fetal sheep and of fetal sheep with diaphragmatic hernias. The normally occurring hypoplasia was at least partly rescued due to a tracheal occlusion (33, 34).

Thus, there is firm evidence that the lung liquid in the lumen is under pressure and that a given pressure difference between the lumen and the surrounding tissue is essential to normal lung growth. The airways are able to resist this pressure—as spontaneous narrowing indicates—and we hypothesize that it is the tone of the airway smooth muscle principally responsible in maintaining this intraluminal pressure. Without this tone a compliant airway wall would be likely to distend as lung liquid is secreted. However, the terminal sacs of the epithelial tubules in the very early pseudoglandular stage are more likely to yield due to the lack of a musculature. Other static forces in the lung may also play a role, but in general they are small during early and midgestation. In rabbits an outward recoil pressure does not develop until late in gestation. Lung compliance doubles during the third trimester (35). The spontaneous contractions produce a rhythmic mechanical stimulus in these highly compliant tissues, which, as we propose, contributes to a normal airway differentiation and branching. But the rhythmic forces the musculature generates are not limited to these highly compliant tissues. It also generates forces across the airway wall and adjacent parenchyma which could well provide the stimulus to growth factor production via mechanotransduction (9, 36), a suggestion supported by the finding that a pulsatile stimulus is more effective than a static one in stimulating lung growth *in vitro* (37, 38).

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