Effect of unilateral diaphragmatic paralysis on postpneumonectomy lung growth

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In many mammalian species, removal of one lung (pneumonectomy, PNX) results in the compensatory growth of the remaining lung. Although the potential importance of oxygen concentration (23), blood flow (5), and growth factors (20) to post-PNX growth have been reported, parenchymal stretch (14) is generally considered the major factor controlling compensatory growth.

The evidence for mechanical stretch regulating post-PNX lung growth is based on several indirect observations. Foremost, a reproducible experimental observation is that preventing stretch of the post-PNX lung, by packing of the residual intrathoracic space with inert material, limits compensatory growth (2, 3, 6, 13). Conversely, increasing the post-PNX stretch appears to increase compensatory growth. Maneuvers such as increasing the amount of lung resected (right lung vs. left lung) (15) and ex vivo overexpansion of the isolated perfused lung (22) produce responses consistent with enhanced growth.

The importance of respiratory muscle-associated stretch to lung growth is also suggested by several observations in development. Fetal breathing movements stimulate cellular proliferation and growth (16). The absence of expansion, due to diaphragmatic hernia or oligohydramnios, causes pulmonary development to cease (12). Similarly, in both humans (4) and animal models (1, 25, 26), normal intrapartus lung development appears to depend upon intact innervation of the diaphragm. In postnatal animals, phrenic nerve transection (PNT) results in compromised lung growth (19, 21, 24). Despite these data suggesting the relevance of stretch in pre- and postnatal lung growth, little is known about the contribution of respiratory muscle function to adult lung growth.

To test the hypothesis that diaphragmatic activity contributes to post-PNX lung growth, we studied post-PNX lung growth in adult mice. Our results suggest that diaphragmatic activity directly contributes to compensatory lung growth.

METHODS

Mice. C57/B6 mice (Jackson Laboratory, Bar Harbor, ME), 8–12 wk old and 22–30 g, were used in all experiments. The care of the animals was consistent with guidelines of the American Association for Accreditation of Laboratory Animal Care (Bethesda, MD) with the protocol reviewed and approved by the Harvard Medical School Institutional Animal Care and Use Committee.

Anesthesia and intubation. The animals were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 6 mg/kg xylazine (Phoenix Scientific, St. Joseph, MO). The animals were intubated under direct vision with a standard 20-gauge Angiocatheter (BD Insyte, Sandy, UT) for the PNX procedure. An 18-gauge Angiocatheter (BD Insyte) was used for forced oscillation measurements. The general anesthesia was performed using a FlexiVent rodent ventilator (SciReq, Montreal, QC Canada). Ventilator rates of 200/min, tidal volume (VT) of 10 ml/kg with positive end-expiratory pressures of 3 cmH2O, and a pressure limit of 30 cmH2O were used.

Pulse oximetry. The MouseOx (STARR Life Sciences, Oakmont, PA), a pulse oximeter with a proprietary plethysmographic waveform analysis algorithm optimized for rats and mice (9), was placed on the shaved skin overlying either the neck or proximal thigh. MouseOx data file exported to Excel for analysis demonstrated oxygen saturation in all surgical groups greater than 95% during and after perioperative oxygen supplementation.

PNX and PNT. In all mice, the left lung was exposed with a fifth intercostal space thoracotomy, and the hilum was ligated with a 5–0 silk tie (Ethicon, Somerville, NJ). In PNT mice, the phrenic nerve was identified on the left lateral pericardium. The nerve was mobilized with gentle traction, producing diaphragmatic tenting, confirming its identity. The nerve was sharply transected. A recruitment maneuver (named “TLC” by SciReq) was performed while closing the thoracotomy. The animals were removed from the ventilator and extubated.
upon recovery of spontaneous ventilation. Bilateral ink markers were placed at the costal margins in the midclavicular line. Digital video of the marker movement was routinely recorded on emergence from anesthesia. The animals were recovered with supplemental oxygen and external warming; Subcutaneous 2.4 mg Buprenorphine (Hospira, Lake Forest, IL) was administered two times daily for 48 h.

Lung weights. The blood-free lung wet weight was obtained after exsanguination. With a beating heart, a cavall venotomy was performed, and the intravascular blood volume drained into an absorptive pad as previously described (17). The weight of the whole lung, as well as individual lobes, was expressed as a ratio of lung weight to animal weight. The lung weight index (LWI), grams per kilogram body weight, was calculated to correct for variability in animal size.

Lung volumes. Lung volumes were measured using the Flexivent (SciReq) rodent ventilator (8). The animals were hyperventilated at a rate of 300/min to remove spontaneous respiratory drive. The animals were allowed to acclimate to the ventilator for 2 min before standardization of the volume history with three consecutive total lung capacity maneuvers using a 3-s ramp to a 30 cmH2O hold. The mean of three measures was taken per animal, with measurements accepted at a minimum coherence of 0.89; consistently high coherence suggested input impedance with a low signal-to-noise ratio.

CT scanning. MicroCT scans were obtained with a GE eXplore 120 CT scanner at 50 μm/pixel resolution. A pressure-limited ventilator with a rate of 50/min was used during CT scanning; animals were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine and 6 mg/kg xylazine. Cranial and caudal endpoints were defined, and the animal was imaged at two shots per gantry turn, gating on peak inspiration and expiration. Upon completion of imaging, the animal recovered in a warmed cage. Image analysis and three-dimensional reconstructions were performed with the OsiriX software (www.osirix-viewer.com).

Statistical analysis. Data analysis was performed using XLstat (Addinsoft, New York, NY) add-in for Microsoft Excel. Results reflected the mean ± 1 SD unless otherwise noted. Statistical significance is determined using analysis of variance with a P value of 0.05. Pairwise comparisons were performed as needed using Tukey’s test.

RESULTS

Ventilatory effects of PNT. After PNT, the reduced spontaneous breathing rate associated with sedation enabled the visual tracking of costal margin excursion. Measured in the midclavicular line, ventilatory movement was markedly reduced after PNT (Fig. 1). The ipsilateral reduction in costal margin excursion was similar in all PNT groups; however, the contralateral (compensatory) excursion was greater in the PNX group (data not shown). Furthermore, the ventilatory asymmetry was persistent at all subsequent time points studied (1–21 days). To assess the physiological response to PNT, oximetry demonstrated oxygen saturations uniformly greater than 95%.

To assess breathing mechanics after PNT, plethysmography demonstrated a decrease in VT and MV during the initial postoperative 48 h, the period of parenteral analgesia. From days 3 to 21, there was no significant difference between the PNX animals (PNX and PNX + PNT) in VT, frequency, and MV (P > 0.05) (Fig. 2).

Effect of PNT without PNX. To establish the baseline effect of diaphragmatic paralysis on lung volume and lung weight, PNT was compared with sham thoracotomy alone. After 21 days, a Flexivent TLC maneuver (3-s ramp to 30 cmH2O hold) demonstrated that the volume of the lungs in the PNT mice was lower than the sham thoracotomy mice (P < 0.05) (Fig. 3). The lower lung volumes persisted despite additional recruitment maneuvers (data not shown). Similarly, the LWI was lower in the PNT mice compared with sham thoracotomy mice; most of the weight difference was associated with the ipsilateral left lung (P < 0.05) (Fig. 3C). The differentially lower weight index in the ipsilateral lung after PNT, however, was not significant when expressed as a ratio of LWIs (P > 0.05) (Fig. 3D).

Structural mechanics after PNT. To investigate the structural changes of the mouse thorax post-PNT, respiratory-gated microCT imaging was performed 3 days after PNX. In mice with PNX alone (intact phrenic nerve), the cardiac lobe was displaced in the left hemithorax with a broad diaphragmatic interface (Fig. 4, top). In contrast, mice with PNX and PNT demonstrated incomplete displacement and significantly decreased volume of the cardiac lobe (Fig. 4, bottom); the microCT-derived volume of the cardiac lobe was 22% smaller after PNT (P < 0.05). To identify potential functional changes in the lung parenchyma associated with diaphragmatic paralysis, the lungs of post-PNX mice, with and without PNT, were
probed using the forced oscillation technique. Fitting the constant-phase model to respiratory system impedance data produced measures of Newtonian resistance, tissue damping, elastance, and hysteresivity (Fig. 5). Newtonian resistance was elevated in the first week after PNT (*P < 0.05), consistent with an altered structural alignment. Measures of tissue damping, tissue elastance, and hysteresivity, indicating the material properties of the peripheral lung, were unchanged after PNT (*P > 0.05).

Effect of PNT on compensatory growth. To determine the effect of PNT on lung growth after left PNX, age-matched
mice were assigned to the following three groups: 1) no surgery, 2) left PNX, or 3) left PNX with PNT. As assessed by the LWI, compensatory growth was observed in the remaining right lung after left PNX (Fig. 6A). As expected, the cardiac lobe demonstrated the most relative growth (Fig. 6B). In contrast, compensatory growth was nearly completely abrogated by PNT (Fig. 6, A and B). The LWI of individual lobes, assessed on day 7 (Fig. 6C) after surgery, demonstrated that PNX + PNT lobes were statistically indistinguishable from nonsurgical controls (*P > 0.05) and significantly smaller than

Fig. 4. Respiratory-gated microCT scans demonstrating the anatomic configuration of the thorax at peak inspiration. Respiratory gating was used to obtain images at a comparable phase in the respiratory cycle. The lung is shown after PNX without (top) and with (bottom) PNT. The cardiac lobe is pseudocolored red for presentation. Cardiac lobe volumes, based on inspiratory-gated microCT scans, were 22% lower in mice after PNX + PNT (P < 0.05).
lobes from PNX mice ($P < 0.001$). Similarly, the LWI on day 21 (Fig. 6D) after surgery demonstrated that PNX + PNT lungs were statistically indistinguishable from nonsurgical controls ($P > 0.05$) and significantly smaller than lungs from PNX mice ($P < 0.001$).

**DISCUSSION**

PNT provided a unique test of the contribution of cyclic stretch to post-PNX lung growth. PNT selectively eliminated the ipsilateral cyclic stretch after PNX. In contrast to plombage, PNT did not alter the configuration of the left pleural space or entrap the remaining lung. The observation that PNT abrogated the expected increase in post-PNX lung weight indicates that cycle stretch is a controlling factor in compensatory lung growth.

The contribution of cyclic stretch to compensatory lung growth may be particularly relevant after murine PNX because of rodent anatomy. In contrast to the three lobes in the human right lung, rodents have an additional posterior mediastinal lobe, variously referred to as the cardiac, retrocaval, or median lobe. Prior studies have demonstrated that the cardiac lobe is displaced in the left hemithorax after PNX (8, 10). Intriguingly, the cardiac lobe demonstrates the greatest relative increase in compensatory lung growth (17).

In these studies, PNT was associated with altered structural realignment of the cardiac lobe after PNX. Within the first few days after surgery, microCT scanning demonstrated limited displacement and smaller volumes of the cardiac lobe. Consistent with underinflation of the lung parenchyma, and compromised tethering of the airways, airway resistance was elevated in the first week after surgery. These changes, in addition to blunted compensatory growth, were directly linked to the loss of diaphragmatic function.

In contrast to the apparent mechanical consequences of PNT, there were few detectable physiological effects. After the initial perioperative period, there were no observable differences between the sham thoracotomy, PNX, and PNX + PNT experimental groups; that is, the activity level and apparent respiratory pattern of the animals were indistinguishable. Similarly, the postoperative oxygen saturation and MV (except for the initial 48 h) were the same between PNX and PNX + PNT groups.

The potential impact of PNT on the material composition of the peripheral lung was assessed by ventilatory mechanics. Forced oscillation lung impedance measurements, fitted to the constant-phase model (11), provided a measure of central and peripheral lung mechanics. Peripheral tissue resistance, thought to be a measure of frictional losses occurring at the elemental level (7), was indistinguishable between PNX and PNX + PNT groups. These observations indicate that growth abrogation was less an untoward effect of PNT on the material composition of the peripheral lung than the absence of a compensatory growth signal.

An unexpected observation was the similar plethysmographic $V_T$ and respiratory rates in the PNX and PNX + PNT
groups 2 days after PNX. We had anticipated a diminished VT and increased respiratory rate associated with PNT. The absence of a significant difference between groups suggests the relative insensitivity of plethysmographic data to compensatory mechanics after PNT. Our observation that contralateral chest wall excursion was greater after PNT suggests at least one mechanism for maintaining compensatory VT and respiratory rates. We suspect that future advances in dynamic imaging should provide useful insights into these compensatory mechanisms.

In a solitary study of phrenic nerve avulsion in adult rats, Cohn noted an 11–19% decrease in the expected weight of the lungs 14 days after surgery (3). Notably consistent with these findings, we observed an 8–11% decrease in the weight of the left lung after PNT alone (no PNX). These findings suggest that continued diaphragmatic function is important for the ongoing maintenance of proper lung structure. We suspect that these observations may provide evidence of morphostasis, that is, an ongoing process responsible for the maintenance of function and form (18). Based on these observations after PNT, we speculate that parenchymal lung stretch may participate in human lung repair. Similarly, patients with emphysema and pulmonary fibrosis may represent disordered or blunted morphostasis in humans.

Finally, an underappreciated challenge is the experimental confirmation of diaphragmatic paralysis. In contrast to humans, the rapid ventilation rate and sedation-related hypoxemia (9) precluded the use of conventional imaging to demonstrate paradoxical diaphragmatic movement. The most reliable indicator of diaphragmatic paralysis, in our studies, was the asymmetric excursion of the costal margin. Because of the rapid respiratory rate of the awake mouse, ventilatory asymmetry was most reliably assessed during the emergence from general anesthesia; typically, ventilatory asymmetry was assessed soon after extubation. We routinely obtained postoperative video recordings to document PNT. Although incomplete transection of the phrenic nerve was rare, these mice were readily identified during their emergence from anesthesia.

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DISCLOSURES
No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS
REFERENCES


