Regenerative growth of respiratory bronchioles in dogs

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Received 27 October 1999; accepted in final form 16 February 2000

Hsia, C. C. W., X. S. Zhou, D. J. Bellotto, and H. K. Hagler. Regenerative growth of respiratory bronchioles in dogs. Am J Physiol Lung Cell Mol Physiol 279: L136–L142, 2000.—Loss of lung units due to pneumonectomy stimulates growth of the remaining lung. It is generally believed that regenerative lung growth involves only alveoli but not airways, a dissociated response termed “dysanaptic growth.” We examined the structural response of respiratory bronchioles in immature dogs raised to maturity after right pneumonectomy. In another group of adult dogs, we also examined the effect of preventing mediastinal shift after right pneumonectomy on the response of respiratory bronchioles. In immature dogs after pneumonectomy, the volume of the remaining lung increased twofold, with no change in volume density, numerical density, or mean diameter of respiratory bronchiole, compared with that in the control lung. The number of respiratory bronchiole segments and branch points increased proportionally with lung volume. In adult dogs after pneumonectomy, prevention of mediastinal shift reduced lung strain at a given airway pressure, but lung expansion and regenerative growth of respiratory bronchiole were not eliminated. We conclude that postpneumonectomy lung growth is associated with proliferation of intra-acinar airways. The proportional growth of acinar airways and alveoli should optimize gas exchange of the regenerated lung by enhancing gas conductance and mixing efficiency within the acinus.

IT HAS BEEN DEMONSTRATED that regenerative lung growth occurs in immature and mature animals after pneumonectomy. Immediately after lung resection, there is active cellular proliferation in the remaining lung (1, 18, 26), leading to an increase in alveolar surface area and septal tissue components associated with normalization of mechanical and gas-exchange function (2, 3, 7, 24, 25). The number and size of alveoli and alveolar ducts increase to various degrees (2, 19). In immature dogs undergoing right pneumonectomy and subsequently raised to maturity, regenerative alveolar growth returned lung volume and alveolar septal tissue volumes to normal (20), associated with normalization of pulmonary diffusing capacities at rest and during exercise (20, 22). Regenerative alveolar growth also occurs in adult dogs after 55% lung resection by right pneumonectomy (10) but not after 45% resection by left pneumonectomy (9). In contrast to immature dogs, regenerative alveolar growth in adult dogs occurs slowly and incompletely; ultimate gas-exchange function remains abnormal (12, 13). One major factor that might explain the different responses to left and right pneumonectomies in adult dogs is the greater mechanical stretch experienced by the remaining lung after right pneumonectomy. Mechanical stretch stimulates alveolar epithelial cell proliferation in vitro (14), although its in vivo effect on the structural dimensions of the normal or postpneumonectomy lung is unclear.

The basic anatomic unit of pulmonary gas exchange is an acinus, which is the lung distal to a terminal bronchiole and composed of several generations of respiratory bronchioles, leading to alveolar ducts and alveoli. Unlike an alveolar duct, a respiratory bronchiole has a complete wall surrounded by smooth muscle. Openings of alveoli can be seen along the length of a respiratory bronchiole. Gas transport within respiratory bronchioles, alveolar ducts, and alveoli occurs predominantly by the process of diffusion. It is believed that in mammals, airway branching morphogenesis is complete in utero and that postpneumonectomy regenerative lung growth involves predominantly alveoli and alveolar ducts but not conducting airways, a dissociated response termed “dysanaptic growth.” This conclusion is inferred from postpneumonectomy estimates of airway resistance in dogs (4), volume of conducting airways in rats (3), and total cross-sectional area of conducting airways in ferrets (15). It is not known whether respiratory bronchioles, which serve both conducting and respiratory gas-exchange functions, participate in regenerative lung growth. In the present study, we measured the dimensions of respiratory bronchioles in dogs that underwent right pneumonectomy either as immature puppies or as fully mature adults to address two fundamental issues: 1) is regenerative alveolar growth associated with structural changes in the respiratory bronchioles, and 2) does prevention of mediastinal shift and alveolar strain after pneumonectomy alter the structural response of respiratory bronchioles?
METHODS

Animals and Surgery

The experimental protocol was approved by the Institutional Animal Care and Research Advisory Committee.

Immature animals. Purebred litter-matched male foxhounds underwent right pneumonectomy at 2 mo of age (pneumonectomy group; removing the right lung that constitutes 55% of total lung tissue) or right thoracotomy without pneumonectomy (sham group). The techniques of anesthesia and surgery have been described in detail previously (8, 22). Physiological and radiological studies were performed at rest under anesthesia during maturation (22, 23). On reaching somatic maturity at 1 yr of age, dogs were trained to exercise on a treadmill (11). Bilateral carotid artery loops were constructed to allow acute catheterization of the artery and the left ventricle. Gas-exchange and hemodynamic studies were performed during exercise (20, 21). Dogs were euthanized, and postmortem studies were performed at about 18 mo after surgery. Tissue samples for the present analysis were available from four animals in the pneumonectomy group and five animals in the sham group.

Adult animals. Adult male mixed-breed foxhounds underwent right pneumonectomy at 1 yr of age. A customized inflatable silicone prosthesis (CUI Corporation, Carpinteria, CA) was constructed in the exact shape and size of the normal right lung measured at functional residual capacity from serial transverse magnetic resonance images obtained from one dog. The prosthesis was attached to a filling tube on the dorsolateral surface via a reinforced patch. After the right lung was removed at surgery, the prosthesis was placed in the empty right hemithorax. The filling tube was tunneled through the interspace, connected to a subcutaneous injection port, and buried at the nape of the neck. The chest wall was closed in five layers, and the residual air in the thorax was partially aspirated.

In one group of animals, the prosthesis was kept inflated with an equal mixture of air and sulfur hexafluoride (SF₆) to a volume 20% higher than the animal's functional residual capacity measured in the supine position (inflated group). This volume successfully maintained the mediastinum in the midline after pneumonectomy (Fig. 1). The mixture of air with SF₆ retards the rate of gas absorption from the prosthesis. In the other group, the prosthesis was kept deflated (>50 ml; deflated group), allowing mediastinal shift to occur (Fig. 2). The volume of the prosthesis was checked weekly for the first month and then at monthly intervals by helium dilution via the subcutaneous injection port. After each measurement, the prosthesis was refilled to the desired volume, and the position of the mediastinum was checked by chest X ray. The rate of gas loss from the prosthesis was found to be no more than 1.0–1.5 ml·kg⁻¹·wk⁻¹.

After recovery from surgery, animals were trained to run on a treadmill, and physiological studies during exercise were performed (28). Dogs were euthanized, and postmortem studies were performed at about 15 mo after pneumonectomy. Tissue samples for the present analysis were available from five animals in each group.

Euthanasia and Tissue Sampling

The dog was deeply anesthetized with pentobarbital sodium (25 mg/kg iv) and intubated via a tracheostomy. The lungs were collapsed through bilateral intercostal incisions and then inflated within the thorax by the intratracheal instillation of 2.5% glutaraldehyde (buffered in 0.03 M potassium phosphate, pH 7.40, 350 mosM) at a constant hydrostatic pressure (25 cmH₂O) above the highest point of the sternum in the supine position. An overdose of intravenous pentobarbital sodium was given simultaneously. After 60 min of fixation in situ, the lungs and heart were removed en bloc and immersed in 2.5% glutaraldehyde.

The volume of the intact lung was measured by immersion displacement (27). Each lung was sectioned serially at 2-cm intervals, and each cut surface was photographed with 35-mm Ektachrome color film. The volume of the lung after sectioning was estimated from the photographs by point counting with the Cavalieri principle (5). Five tissue blocks (~1 cm³) were selected from the left upper lobe of each animal by a systematic sampling scheme with a random start and embedded in paraffin.

Morphometric Analysis of Respiratory Bronchioles

A video image analysis technique was developed. The tissue block was placed on a Reichert-Jung 2050 microtome mounted on an optical bench; the face of the block was photographed with a Nikon 55-mm micro lens attached to a COHU charged-coupled device solid-state video camera. Surface lighting was provided by fiber-optic white light through a polarized filter. Images of the block face were captured on a Power Macintosh 8500 computer via a QuickCapture image grabbing board (Data Translation). Image analysis was performed with the aid of National Institutes of Health Image software (version 1.60/ppc) using customized macros. Final magnification was approximately ×72. An example of an image is shown in Fig. 3.

Respiratory bronchioles were identified as airways that have openings to alveoli along their walls. Volume density of respiratory bronchioles in lung was determined by point counting. One section from each block was selected at random. Beginning with a random field within this section, 10 nonoverlapping fields were imaged by systematically moving the stage at a fixed interval along the x- or y-axis, yielding a total of 50 images per animal. A test grid was placed over
each image, and the number of points falling on respiratory bronchiole tissue or air within respiratory bronchiole was counted. Volume density of respiratory bronchioles in the lung was calculated as points on respiratory bronchioles divided by total points on lung. Numerical density of respiratory bronchioles was calculated as number of segments or branch points divided by total points on the lung.

Starting from the last field sampled on the above section, 50 serial sections were cut through the block at 20-μm intervals along the z-axis, whereas the x- and y-axes remained fixed. The block face was imaged after each section. The resulting stack of serial images represented a volume of lung of about 4 mm³. Within this defined volume, all respiratory bronchioles were followed through consecutive images and each separate respiratory bronchiole segment was numbered. On each image, the internal contour of every respiratory bronchiole was outlined. The internal diameter of each respiratory bronchiole segment was measured from the minor axis length of its cross-sectional contour on the first and last image on which it was visible, and the measurements were averaged. If a respiratory bronchiole segment was seen along its longitudinal axis, diameter measurement was made at the two ends of the observable length and averaged. The number of segments and branch points of respiratory bronchioles within the stack were counted, and the total number was divided by the volume of the stack to obtain the numerical densities of respiratory bronchiole segments and respiratory bronchiole branch points in lung (in mm⁻³). A volume-weighted average from five blocks was calculated for each animal. The absolute number of respiratory bronchiole segments and branch points in the left lung was obtained from the product of their numerical densities, and the volume of the left lung was measured by the Cavalieri principle. The two methods of estimating numerical density of respiratory bronchiole segments and branch points gave similar results. Data are expressed as means ± SE. Statistical comparison was done by analysis of variance with post hoc comparison by Fisher’s multiple comparison method. We used a commercial software (StatView version 5.0; Statistical Analysis Systems Institute; Cary, NC). P < 0.05 was considered significant.

RESULTS

Body Weight and Lung Volume

Results are shown in Table 1. One animal in the inflated group (dog Z) developed a leak in the prosthesis 9 mo after surgery, and the mediastinum could not be maintained in the midline thereafter. Volume and diffusing capacity of the remaining lung progressively increased over the subsequent months; hence morphometric data from this dog are discussed separately. The adult dogs with an inflated prosthesis had a lower mean body weight than other groups even before undergoing surgery, but body weight did not change significantly after surgery in any of the groups.

After pneumonectomy was performed on either puppies or adults, the volume of the left lung measured by the immersion method increased approximately two-fold compared with that in sham animals. The compensatory increase in lung volume was significantly attenuated in the presence of an inflated prosthesis, but lung volume remained larger than that in the normal left lung in sham animals. Because the lung was fixed under a constant hydrostatic pressure, the volume of the sectioned lung estimated by the Cavalieri principle was consistently lower than that of the intact lung estimated by immersion in all animals in the sham, pneumonectomy, and deflated groups, indicating that alveolar septa were stretched during fixation. However, in the inflated group, lung volume did not change regardless of whether the lung was intact or sectioned; thus in these animals, alveolar septa were not under stretch at the same hydrostatic pressure during fixation.

After pneumonectomy on puppies and on adults in the deflated group, total lung volume was restored to that expected for two normal lungs. In pneumonecto-
mized dogs with an inflated prosthesis, volume of the intact lung was significantly lower than that in two normal lungs, but volume after sectioning was similar to that in dogs with a deflated prosthesis and sham animals.

Volume and Numerical Densities and Diameters

Results are shown in Table 1. Volume density of respiratory bronchioles did not change in the animals pneumonectomized as puppies compared with that in sham control dogs but were significantly higher in dogs pneumonectomized as adults (deflated group) compared with sham control dogs. Presence of an inflated prosthesis was associated with a lower volume density of respiratory bronchioles compared with that in the deflated group. The numerical density of respiratory bronchiole segments in lung, numerical density of branch points in lung, and mean respiratory bronchiole diameters were similar across groups.

Absolute Volume and Number of Respiratory Bronchioles

Results are shown in Fig. 4. In dogs pneumonectomized as puppies, absolute volume of respiratory bronchioles increased 2.3-fold compared with that in the left lung of sham control dogs. The number of respiratory bronchiole segments and branch points increased 2.6- and 2.7-fold, respectively, compared with those in the left lung of sham controls. In dogs pneumonectomized as adults, absolute respiratory bronchiole volume increased 3.3- and 3.6-fold in the inflated and deflated groups, respectively, compared with that in the sham group. The number of respiratory bronchiole segments and branch points increased 2.2- and 1.9-fold in the inflated group and 2.2- and 2.0-fold in the deflated group, respectively, compared with those in the sham group. There was no significant difference in these measurements between inflated and deflated groups.

Table 1. Morphometric data

<table>
<thead>
<tr>
<th></th>
<th>Immature Dogs Raised to Maturity</th>
<th>Adult Dogs With Prosthesis After PNX</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Body wt, kg</td>
<td>30.9 ± 2.3</td>
<td>29.6 ± 1.1</td>
</tr>
<tr>
<td>Left lung volume, ml/kg</td>
<td>60.8 ± 4.3</td>
<td>35.5 ± 2.0</td>
</tr>
<tr>
<td>Total lung volume, ml/kg</td>
<td>60.8 ± 4.3</td>
<td>35.5 ± 2.0</td>
</tr>
<tr>
<td>Mean diameter of RB, µm</td>
<td>262 ± 21</td>
<td>231 ± 26</td>
</tr>
<tr>
<td>Volume density of RB in lung</td>
<td>0.0384 ± 0.0005</td>
<td>0.0402 ± 0.0010</td>
</tr>
<tr>
<td>RB segments in lung</td>
<td>1.583 ± 0.220</td>
<td>1.697 ± 0.316</td>
</tr>
<tr>
<td>RB branch points in lung</td>
<td>0.615 ± 0.091</td>
<td>0.641 ± 0.138</td>
</tr>
</tbody>
</table>

Values are means ± SE. PNX, pneumonectomy; RB, respiratory bronchioles. *P < 0.01 vs. sham. †P < 0.01 vs. deflated. ‡P < 0.01 vs. PNX.

Delayed Mediastinal Shift After Pneumonectomy

In dog Z, in which the inflated prosthesis became deflated 9 mo after pneumonectomy, there was progressive mediastinal shift and volume expansion of the left lung over the subsequent months as documented by serial chest X-rays and physiological measurements (28). At postmortem, intact lung volume of this dog (71.9 ml/kg) was 38% larger than the inflated group mean. As in the inflated group, there was no volume shrinkage after the lung was sectioned (68.1 ml/kg). Volume density (0.0384) as well as numerical densities of respiratory bronchiole segments (0.898) and branch points (0.378) in this dog were all lower than both inflated and deflated group means. Volume density was similar to that in sham animals, whereas numerical densities were ~50–60% of that in sham animals. Mean respiratory bronchiole diameter (338 µm) was intermediate between the inflated and deflated group means. Absolute volume (2.62 ml/kg) and number of respiratory bronchioles segments (61.2 × 10³/kg) and branch points (25.7 × 10³/kg) were 10–20% higher than the mean values in inflated and deflated groups.

DISCUSSION

Structural Basis of Acinar Growth

Structural growth of acini may occur via 1) addition of new acinar units, 2) addition of another generation of acinar airways while the number of acini remains constant, or 3) lengthening and dilatation of existing acinar airways with a corresponding increase in the number of alveoli per unit airway length. Each mechanism could explain the compensatory increase in alveolar air and tissue volumes seen after pneumonectomy but should lead to distinct morphometric characteristics as well as functional consequences in diffusion that are experimentally verifiable. The expected morphometric changes from each type of structural response are as follows.
If postpneumonectomy alveolar growth is not associated with any structural change in respiratory bronchioles, the number of respiratory bronchiole segments and branch points in the remaining lung would not change while the volume of the remaining lung increases. Hence the volume and numerical densities of respiratory bronchioles and numerical density of respiratory bronchiole branch points should be reduced by about 50%. The absolute volume of respiratory bronchioles, number of respiratory bronchiole segments and branch points as well as the mean diameter of respiratory bronchioles should remain unchanged from normal.

If postpneumonectomy lung growth is not associated with growth of respiratory bronchioles and the respiratory bronchioles simply become proportionately distended with expansion of the remaining lung, the mean diameter of respiratory bronchioles should increase while the volume density of respiratory bronchioles remains relatively unchanged. The numerical density of respiratory bronchiole segments and branch points should decrease by 50%. The absolute number of respiratory bronchiole segments and branch points should remain unchanged from that in the control left lung.

If postpneumonectomy alveolar growth is associated with proliferation of respiratory bronchioles without a change in their dimensions through adding either new acinar units or one more generation of respiratory bronchioles, the volume density, numerical density, and diameter of respiratory bronchioles should remain normal. The absolute volume and number of respiratory bronchiole segments and branch points in the left lung should be increased after pneumonectomy. A combination of elongation-dilatation and proliferation should result in intermediate values.

**Response of Immature Dogs Raised to Maturity After Pneumonectomy**

Our data show that in dogs pneumonectomized as puppies the numerical density of respiratory bronchiole segments and branch points are not different from that in the control left lung. The mean diameter of respiratory bronchioles is similar between the two groups. The absolute volume of respiratory bronchioles and the number of respiratory bronchiole segments and branch points in the remaining lung increased significantly compared with those in the same lung in control animals and are similar to those in both lungs of control animals. Hence there has been addition of new respiratory bronchioles after pneumonectomy, occurring either by proliferation of new acini or by proliferation of one more generation of respiratory bronchioles, restoring in either case the number of respiratory bronchioles to normal. Structural adaptation of the respiratory bronchiole parallels that of the alveoli (20), indicating a proportionate growth involving all components of the acinus.

**Response of Adult Dogs to Pneumonectomy**

In both adult groups, the numerical density of respiratory bronchiole segments and branch points is consistently lower than in dogs operated on as puppies; nonetheless, these measurements are still considerably greater than 50% of those in sham animals. The absolute number of respiratory bronchiole segments and branch points is significantly increased from that in the control left lung and similar to that in both lungs of sham animals, indicating that regenerative growth of respiratory bronchioles has occurred. In both adult groups, the diameter of respiratory bronchioles was consistently greater than that in dogs operated on as puppies. The volume density and absolute volume of respiratory bronchioles are also higher, indicating dilatation of respiratory bronchioles. Hence adult dogs show a mixed pattern involving both dilatation and proliferation of respiratory bronchioles after pneumonectomy. This pattern of adaptation also parallels the alveolar response in adult dogs (10).
**Effect of Preventing Lung Expansion After Pneumonectomy**

Despite a lower body weight, dogs with an inflated prosthesis show similar mass-specific lung volumes as dogs with a deflated prosthesis. Lung tissue is normally fixed under tension at a distending pressure of 25 cmH₂O. Tension does not develop in the inflated group, evidenced by the lack of volume change when the intact lung is later sectioned; thus the inflated prosthesis, by maintaining the mediastinum in the midline, has successfully restricted mechanical lung stretch at a given distending pressure. However, the inflated prosthesis did not completely prevent the increase in lung volume after pneumonectomy. The volume of the intact remaining lung in the inflated group is still significantly larger than that in a normal left lung because the remaining lung can still enlarge in the caudal direction by depressing the left hemidiaphragm. Caudal expansion of the remaining lung has been shown by our group (28) in the present animals with computed tomography scan and by Olson and Hoffman (17) in pneumonectomized rabbits with wax plombage. In fact, the volume of the remaining lung not under tension is not altered by the presence of an inflated prosthesis. This is a novel and important observation; i.e., the remaining lung does not expand simply to fill an empty space in response to the increased intrathoracic pressure. Lung volume increases even when mechanical lung strain is minimized, suggesting the existence of other intrinsic signals that stimulate the remaining lung to expand even when space is not readily available.

The lower volume density of respiratory bronchioles in the inflated group is likely due to the slightly smaller diameter compared with that in the deflated group. The absolute number of respiratory bronchiole segments and branch points and the absolute volume of respiratory bronchioles are not different between inflated and deflated groups; hence prevention of mediastinal shift after pneumonectomy does not eliminate the compensatory structural response of respiratory bronchioles.

Data from dog Z are illuminating in showing that delayed mediastinal shift 9 mo after pneumonectomy leads to rebound lung expansion, resulting in a final lung volume that is much larger than expected. The associated dilatation of respiratory bronchioles is an expected response to the increased lung strain. The absolute number of respiratory bronchiole segments and branch points in this dog is only slightly higher than that in pneumonectomized adult dogs with continually inflated or deflated prosthesis. These data support the preservation of structural adaptability even months after pneumonectomy, although further long-term studies are required to determine whether delayed onset of lung strain has the same stimulatory effect on acinar growth as immediate and sustained lung strain.

**Other Growth Signals**

Additional stimuli likely involved in postpneumonectomy lung growth include pulmonary vascular congestion or endothelial shear. After pneumonectomy, the entire cardiac output flows through the remaining lung, effectively doubling pulmonary perfusion per lung unit. Haworth et al. (6) found that ligation of the left pulmonary artery and ductus arteriosus in newborn pigs reduces the size but not the number of alveoli in the left lung 6 mo later. On the other hand, unilateral pulmonary artery ligation increases alveolar size and accelerates alveolar proliferation in the contralateral lung, suggesting a perfusion-related stimulation of alveolar growth. McBride et al. (16), however, showed in ferrets that restriction of pulmonary perfusion by banding one lobar pulmonary artery had little effect on postpneumonectomy weight, volume, and protein and DNA contents of either the banded or the unbanded remaining lobes. In both of these studies, changes in bronchial blood flow were not controlled. The reduction of perfusion to one lung or lobe is counteracted by a correspondingly increased perfusion to the other lung or lobe, so potential interlobar interactions could have obscured any perfusion-related effect. In addition, the 3-wk follow-up period in the study by McBride et al. (16) may not have been long enough to detect evidence of perfusion-induced lung growth.

In conclusion, our results show for the first time that respiratory bronchioles, like alveoli, are capable of regenerative growth after unilateral lung resection. This regenerative response is not eliminated when postpneumonectomy mediastinal shift is prevented, suggesting that signals other than lung strain, such as vascular congestion or endothelial shear, also play an important role. The occurrence of proportional growth of acinar airways and alveoli after pneumonectomy should enhance gas conductance and mixing efficiency within the acinus, thereby optimizing gas exchange in the regenerated lung.

This project was supported by National Heart, Lung, and Blood Institute Grants RO1-HL-45716, HL-40070, and HL-54060. X. S. Zhou was supported by a fellowship from the Will Rogers Memorial Foundation. This work was done during the tenure of C. C. W. Hsia as an Established Investigator of the American Heart Association.

**REFERENCES**


