Long-term post-pneumonectomy pulmonary adaptation following all-trans-retinoic acid supplementation

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MAJOR LUNG RESECTION BY PNEUMONECTOMY (PNX) stimulates compensatory growth of the remaining lung, characterized by balanced increases in the volume of all alveolar air and tissue compartments associated with partial to complete normalization of lung diffusing capacity at a given pulmonary blood flow (7, 29). Of interest, all-trans-retinoic acid (RA), an active metabolite of all-trans-retinol (vitamin A) that modulates the transcription of pleiotropic genes regulating cell proliferation, apoptosis, and differentiation (40), has been reported to enhance postnatal alveolar formation and growth (6, 15) as well as abrogate emphysematous alveolar destruction in rodents (16). To determine if RA enhances post-PNX compensatory lung growth in large animals, we previously administered supplemental RA or placebo for 4 mo to adult dogs following resection of 58% of lung mass by right PNX. Compared with placebo treatment, supplemental RA induced significant, although modest, morphological changes in the remaining lung with greater increases in the volumes of alveolar tissue, interstitium, and capillary endothelium but without a greater increase in lung volume (38). The cellular changes were associated with septal crowding and altered capillary morphology with a higher prevalence of double-capillary profiles reflecting attempted neocapillary formation, but without any improvement in lung diffusing capacity measured at rest under anesthesia (3). In adult dogs following less extensive resection of 42% of lung mass by left PNX, which is not associated with significant whole lung compensatory growth, RA supplementation had no effect on alveolar structure or function (37). These results led us to conclude that supplemental RA modifies some aspects of active alveolar septal growth but cannot induce alveolar growth de novo; because RA stimulates only some components of the septum, the unbalanced response leads to septal distortion that prevented overall functional improvement.

These earlier studies were performed 4 mo following PNX and RA supplementation, i.e., during the period of active compensatory cellular growth, hypertrophy, and matrix deposition. However, post-PNX compensation also involves progressive alveolar remodeling whereby the double capillaries transition into more single capillaries and the alveolar-capillary barrier becomes thinner to facilitate diffusive oxygen uptake (8, 29). Exogenous RA being lipid-soluble is stored in fat tissue and slowly released following the period of supplementation. Therefore, we hypothesized that in addition to the early post-PNX effects on cellular growth, RA supplementation promotes progressive alveolar remodeling leading to long-term functional enhancement. To examine this issue, we administered supplemental all-trans RA or placebo (38) to adult dogs for 4 mo following right PNX. Animals were trained to run on a treadmill and followed for 16 mo after discontinuation of supplementation (20 mo post-PNX). Lung function was measured from rest to exercise and lung structure was quantified under light and electron microscopy. If our hypothesis is true, we expected to find greater balanced increases in alveolar septal tissue and blood components associated with normal septal ultrastructure in animals 20 mo post-PNX (16 mo after RA treatment) compared with placebo and greater progressive improvement in lung function between 4 and 20 mo post-PNX.
METHODS

Animal procedures. Our Institutional Animal Care and Use Committee approved the protocol. Twelve litter-matched purpose-bred male foxhounds (~10 mo old) underwent right PNX (~58% lung removed). Briefly, the animal was fasted overnight; premedicated with buprenorphine, acepromazine, and glycopyrrolate; anesthetized with isoflurane; and intubated. Rectal temperature, heart rate, and transcutaneous oxygen saturation were monitored continuously. The right lung was exposed via a lateral thoracotomy through the fifth intercostal space. Blood vessels of each lobe were dissected free, tied with silk ligature, and cut. The right main stem bronchus was stapled and cut. The bronchial stump was over-sewn with loose mediastinal tissue for added protection and then immersed under saline to check for leaks. The chest wall was closed in five layers. Topical lidocaine was applied to the intercostal nerve and muscle during closure. Residual air in the right hemithorax was evacuated. Buprenorphine was administered postoperatively at regular intervals for analgesia for 2 days and as needed thereafter. An antibiotic was administered for 7 days.

RA supplementation. Beginning 1 day after PNX, animals were divided into two litter-matched groups. The RA group received all-trans-RA (Sigma, St. Louis, MO) 2 mg/kg per day, 4 days per wk for 4 mo. The drug was dissolved in a small amount of alcohol in a light-protected environment per the manufacturer’s instruction. The dissolved drug was suspended and thoroughly mixed in 1 ml of vegetable oil. The placebo control group received only the oil diluent. The prepared drug or placebo was mixed with 1 tablespoon of peanut butter, labeled in a blinded fashion, and immediately given to separate personnel who administered it to the animal either in a bowl or spoon-fed into its mouth. The animal was observed until all the peanut butter had been swallowed. A drug holiday of 3 days/wk was provided to minimize induction of metabolism. On the basis of our previous canine studies (37, 38), this regimen of RA supplementation elicited significant post-PNX cellular, morphological, and physiological responses without incurring toxicity. The staff administering the drug or placebo were blinded.

Two animals in the placebo group died unexpectedly. One animal developed post-PNX pulmonary edema; previously undiagnosed congenital cardiac valvular abnormalities were discovered at necropsy. A second animal suffered cardiac arrest on induction of anesthesia for an unrelated dental procedure; no definitive pathology was found at necropsy. Thus six animals in the RA group and four in the placebo group completed all the studies. Timeline is shown in Fig. 1.

Resting lung function. Resting lung function was measured at three time points: 1) 2 mo before surgery, 2) 4 mo post-PNX (at the end of RA supplementation), and 3) 16 mo post-PNX (1 yr after the end of RA supplementation). Lung function was measured supine under anesthesia by a previously described rebreathing method (30). The animal was anesthetized and intubated with a cuffed endotracheal tube and mechanically ventilated (model 607, Harvard Apparatus, Millis, MA) at 8–12 ml/kg tidal volume and a rate sufficient to suppress spontaneous ventilation. A latex balloon-tipped catheter was inserted into the distal one-third of the esophagus to measure esophageal pressure changes. Mouth pressure, rectal temperature, heart rate, and transcutaneous oxygen saturation were monitored. The endotracheal tube was connected to a manifold, allowing the animals to be connected to either the ventilator or a 3-liter calibrated syringe containing the desired inspiratory gas mixture.

Pressure-volume relationship. Pressure-volume (PV) curves were measured at four time points: pre-surgery and 4, 16, and 20 mo post-PNX. From end-expiratory lung volume (EELV), the lungs were inflated with 15, 30, 45, or 60 ml/kg of air and held for 8 s. Transpulmonary pressure was calculated as the difference between esophageal and mouth pressures. The inflation volume was incremented and then decremented stepwise; duplicate measurements at each lung volume were averaged. PV curves were analyzed by the exponential model of Salazar and Knowles (26) with the statistical method of Pengelly (19). The derived constants were used to estimate lung volume at predetermined transpulmonary pressures and compared between groups by repeated-measures ANOVA.

Rebreathing measurements at rest. Lung volumes, cardiac output, diffusing capacity for carbon monoxide (DLCO) and nitric oxide (DLNO), and septal (tissue plus capillary blood) volume were measured by a rebreathing technique (7). The test gas mixture, consisting of 0.3% CO, 0.3% methane, 0.6% acetylene, either in 21% O2 and a balance of N2 or in 99% O2, was humidified and drawn into a 6-liter Mylar reservoir bag. Prior to each measurement, NO (40 ppm) was added to the reservoir bag and thoroughly mixed. The desired volume of test gas mixture (30 or 45 ml/kg plus apparatus dead space) was drawn into the 3-liter calibrated syringe. The animal was first ventilated with the appropriate background gas (21 or 100% O2) for 2 min to ensure equilibration with resident alveolar gas. At a selected end expiration, the expiratory port was occluded. Following lung expansion with three cumulative tidal breaths and passive deflation to EELV, the test mixture was delivered and rebreathed in and out of the syringe for 16 s at a rate of 30 breaths/min. Gas concentrations were continuously monitored at the mouth by a chemiluminescence NO analyzer (Sievers NOA280, GE Analytical Instruments, Boulder, CO), an infrared gas analyzer for CO, methane and acetylene (Sensors, Saline, MI), and a mass spectrometer for O2, CO2, and N2 (1100 MGA, Perkin-Elmer, Norwalk, CT). Analyzers were calibrated each study day according to manufacturer’s specification. Rebreathing measurements were obtained in random order at two inspired O2 concentrations (21 and 99% O2) at two lung volumes (30 and 45 ml/kg above EELV). Duplicate measurements under each condition were averaged.

A venous blood sample was drawn before and at the end of the experiment to measure hemoglobin and carboxyhemoglobin concentrations (OSM3, Radiometer, Copenhagen, Denmark). Hematocrit was measured using a capillary tube centrifuge. Lung volumes (BTPS) were calculated from methane dilution, cardiac output from the exponential disappearance of end tidal acetylene with respect to methane, corrected for the intercept of CO disappearance (25), and DLCO and DLNO from the exponential disappearance of end tidal CO and NO, respectively, with respect to methane (24, 31). End-tidal points were selected from the log linear portion of the disappearance curves; the first three breaths and those after 12 s were discarded. From DLCO measured at two alveolar O2 tensions (PACO2 in mmHg), we estimated membrane diffusing capacity (DMCO) and pulmonary capillary blood volume (Vc) by the Roughton-Forster model (23):
where $\theta_{CO}$ is the empirical CO uptake by whole blood at 37°C [in ml CO-(mmHg ml blood)$^{-1}$] calculated from mean PAO$_2$, during rebreathing and hemoglobin concentration ($\text{Hb}$) in g/dl:

$$
\frac{1}{D_{CO}} = \frac{1}{D_{Ms}} + \frac{1}{\theta_{CO}} \cdot \frac{1}{V_c}
$$

(1)

where $\beta$ is a function of rectal temperature (RT, in°K):

$$
\beta = 0.00517e^{10\left(\frac{RT - 310}{310}\right)}
$$

(3)

Lung fixation. After completion of terminal physiological measurements, a tracheotomy was performed under deep anesthesia. A cuffed endotracheal tube was inserted and tied securely. The abdomen was opened via a midline incision, and a rent was made through each hemidiaphragm to collapse the lung. The ventilator was disconnected, and the lung reinfated within the thorax by tracheal instillation of 2.5% buffered glutaraldehyde at 25 cmH$_2$O hydrostatic pressure above the sternum. An overdose of Euthasol was simultaneously administered to stop the heart. Following instillation, the endotracheal tube was closed to maintain airway pressure. The lung was removed intact and immersed in buffered 2.5% glutaraldehyde in a plastic bag, floated on a water bath, and stored at 4°C until further processing.

Lobar volume. The right lung was separated into the cranial, middle, and caudal lobes. The cranial lobe was sectioned into a caudal part of the cranial lobe, was often completely separated. Volume of each intact lobe was first measured by saline immersion. Then each lobe was sectioned serially at 1 cm intervals, and the cut surfaces photographed (Nikon Coolpix 995). Volume of each sectioned lobe was estimated using the Cavalieri principle (39); this tension-free volume was used in subsequent morphometric calculations.

Lung sampling and morphometric analysis. Each lobe was sampled separately using an established four-level stratified scheme (35): gross (level 1, $\times \sim$2), low-power light microscopy (LM; level 2, $\times$275), high-power LM (level 3, $\times$550), and electron microscopy (EM; level 4, $\times$19,000). For level 1, photographs of serial sections were analyzed by point counting using standard test grids to exclude structures larger than 1 mm in diameter, yielding an estimate of the volume density of coarse parenchyma per unit of lung volume ($V_{v(cp,l)}$). For level 2, four tissue blocks were selected per lobe using a systematic sampling scheme with a random start. Blocks were embedded in glycol methacrylate, sectioning ($5 \mu$m) and staining with toluidine blue. One section per block was overlaid with a test grid. From a random start, at least 10 non-overlapping microscopic fields were systematically sampled at $\times$275. Using point counting, structures between 20 $\mu$m and 1 mm in diameter were excluded to estimate the volume density of fine parenchyma per unit volume of coarse parenchyma ($V_{v(fp,cp)}$).

For levels 3 and 4, four blocks per lobe were sampled using a systematic random scheme, postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, treated with 2% uranyl acetate, dehydrated through graded alcohol, and embedded in Spurr (Electron Microscopy Sciences, Hatfield, PA). Each block was sectioned ($1 \mu$m) and stained with toluidine blue. One section per block was overlaid with a test grid. From a random start, at least 20 non-overlapping microscopic fields per block were systematically imaged (80 images/lobe) to estimate the volume density of alveolar septa per unit volume of fine parenchyma ($V_{v(s,fp)}$) by excluding all structures exceeding 20 $\mu$m in diameter (level 3).

For EM analysis (level 4), two blocks per lobe were sectioned (80 nm thickness) and mounted on copper grids. Each grid was examined under transmission electron microscopy (JEOL EXII) at $\times \sim$19,000. Thirty non-overlapping EM fields per grid (60 images/lobe) were sampled systematically from a random start. Images were captured with a charge-coupled device camera (Gatan, model C73-0200) and a high-resolution monitor. Segal cells were identified by their typical morphological characteristics. The volume densities of epithelium (types I and II), interstitium, and endothelium were estimated by point counting. Alveolar epithelial and capillary surface densities were recalibrated to read in the parts per million (ppm) range, and duplicate section imaging measurements were made at 30 and 45 ml/kg as described above. Alveolar NO concentration was measured from the average filtered signal during exhalation following rebreathing of 100% O$_2$ for 20 s. Alveolar NO output was calculated from the end-rebreathing NO partial pressure multiplied by DL$_{NO}$ since at equilibrium exhaled NO output equals the amount of NO carried away by capillary blood (11, 20).

Rebreathing measurement during exercise. These methods are well documented (7, 29). A rebreathing bag was prefilled with a volume of test gas equal to (average tidal volume $\times$ ATPD). The gas exchange septal tissue volume was estimated from the extrapolated intercept of the acetylene disappearance curve to zero time; replicate estimates in each animal were averaged.

Exercise breathing circuit. A customized, respiratory mask was constructed for each animal (2) and connected to a large two-way non-rebreathing valve (Hans Rudolph, model 2700). Inspired and expired tidal volumes were measured by separate screen pneumotachographs (Hans Rudolph model 3813). Expired gas concentrations were sampled continuously by a mass spectrometer distal to a mixing chamber. Minute ventilation, O$_2$ uptake, CO$_2$ output, and respiratory rate were averaged every 10 breaths. Electrocardiogram and rectal temperature were continuously recorded.

Exercise training. The physical training program is well established (9). Each animal was familiarized with the motor-driven treadmill over 1–2 wk. Training consisted of running for 30 min/day, 5 days/wk at a workload equivalent to 60–80% of maximal oxygen uptake until reproducible levels of O$_2$ uptake were obtained at each speed and incline and a maximum speed and incline was established beyond which exercise could not be sustained for 5 min. Training began before surgery, resumed 3 wk after surgery, and continued two or three times per week throughout the study.

Peak oxygen uptake. The external jugular vein was cannulated under local anesthesia on the day of study for blood sampling. Peak O$_2$ uptake was determined by an incremental exercise protocol. After 5 min of warm-up at 6 mph and 0% grade, the treadmill speed was increased to 8 mph $\times$ 3 min; then the treadmill grade was incremented by 5% every 3 min until a plateau in O$_2$ uptake was reached or until volitional termination. A blood sample (3 ml) was drawn each minute under local anesthesia on the day of study for blood sampling. Peak oxygen uptake, O$_2$ uptake, and respiratory exchange ratio were measured during exercise by the open circuit method with toluidine blue. One section per block was overlaid with a test grid. From a random start, at least 10 non-overlapping microscopic fields were systematically sampled at $\times$275. Using point counting, structures between 20 $\mu$m and 1 mm in diameter were excluded to estimate the volume density of fine parenchyma per unit volume of coarse parenchyma ($V_{v(fp,cp)}$).
estimated by intersection counting. At least 300 points or intersections were counted per grid, yielding a coefficient of variation below 10%. The length of test lines (l) that transect the barrier from the epithelial surface to the nearest red cell membrane were measured to calculate harmonic mean thickness of the tissue-plasma barrier ($\tau_{th}$). Morphometric data were calculated for each lobe, and a volume-weighted average for the entire lung was obtained. Absolute volume and surface area of individual structures were obtained by relating the volume and surface densities at each level back through the cascade of levels to the volume of the lobe (35).

Morphometric estimates of lung diffusing capacity for O$_2$ (DL$_{CO}$) were calculated using an established model (36) that describes the gas diffusion path from alveolar air to capillary hemoglobin binding sites as two serial conductance across the combined tissue-plasma barrier (D$\bar{b}_{O2}$) and capillary erythrocytes (D$\bar{b}_{DeO2}$).

To quantify the prevalence of single- and double-capillary profiles (38), two EM grids from each lobe were systematically and completely sampled ($\times$1,000) with a test grid and the number of single and double capillary intercepts counted, resulting in over 200 total profiles per animal.

**Statistical analysis.** Results were normalized by body weight and expressed as means ± SD. Comparison between groups was performed by unpaired t-test or one-way ANOVA. Temporal effects were compared by repeated-measures ANOVA. Diffusing capacities and their components were plotted with respect to pulmonary blood flow and compared by repeated-measures ANOVA. Since we previously showed significant stimulation on septal cellular components after 4 mo of post-PNX RA supplementation (38), a one-tailed $P$ value of 0.05 or less between groups was considered significant.

**RESULTS**

**Physiological measurements.** Body weight and hemoglobin concentration increased ~20% in both groups during the course of the study. The expected post-PNX compensatory increases (13, 30) in lung volumes, DL$_{CO}$, DL$_{NO}$, DM$_{CO}$, and Vc of the remaining lung were again observed (Table 1). However, there were no significant differences in lung function between RA and placebo treatment groups at any time point (Table 1).

Combined data from all animals showed significant temporal changes; total resting pulmonary blood flow decreased progressively post-PNX. By 4-mo post-PNX, lung volume and diffusing capacities had largely returned to pre-PNX levels and remained normal 16 mo post-PNX although increased from corresponding values at 4 mo post-PNX. Septal (tissue + blood) volume measured by acetylene uptake was slightly lower at 4 mo post-PNX compared with pre-PNX; the RA group partially recovered with time but both groups were still lower than pre-PNX at 16 mo. Static pressure volume curves (Fig. 2) showed a similar slight downward shift in both groups at 4 mo post-PNX, which recovered by 16 mo.

During light exercise (7 mph, 0% grade) tidal volume was higher in the RA group vs. placebo. At peak exercise, pulmonary blood flow was lower in RA vs. placebo. There were no other significant differences between RA and placebo groups in lung function from rest to peak exercise (Table 2); the relationships of DL$_{CO}$, DM$_{CO}$, and Vc with respect to pulmonary blood flow were not significantly different between groups (DL$\bar{c}_{O2}$ shown in Fig. 3).

**Morphometric results.** Representative alveolar morphology under LM and EM in the two experimental groups is shown in Fig. 4. Quantitative data are summarized in Tables 3–5. In RA-treated lungs compared with placebo, volume of the fixed lung was unchanged (Table 3). Morphometric capillary hematocrit was slightly lower. Whole lung arithmetic mean septal thickness was not significantly different. Thickness of the capillary basal lamina was 29% higher. The average harmonic mean thickness of the blood-gas barrier ($\tau_{th}$) (Table 3) and the frequency distribution of individual $\tau_{th}$ (1/intercept length) measurements shifted toward higher values (Fig. 5), indicating increased barrier resistance to diffusion. The prevalence of double septal capillaries was significantly (82%) higher.

Volume densities and surface densities of alveolar components, expressed with respect to total lung volume, are

<table>
<thead>
<tr>
<th>Table 1. Physiologic measurements at rest</th>
<th>Pre-PNX</th>
<th>4 mo Post-PNX</th>
<th>16 mo Post-PNX</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Placebo</strong></td>
<td><strong>RA</strong></td>
<td><strong>Placebo</strong></td>
<td><strong>RA</strong></td>
</tr>
<tr>
<td>Body wt, kg</td>
<td>20.3 ± 2.1</td>
<td>22.6 ± 2.0$^b$</td>
<td>23.8 ± 2.6$^b$</td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>9.7 ± 0.8</td>
<td>11.5 ± 0.4$^b$</td>
<td>11.8 ± 0.6$^b$</td>
</tr>
<tr>
<td>Anesthetized</td>
<td>12.0 ± 1.0</td>
<td>13.2 ± 0.8</td>
<td>14.1 ± 0.9$^b$</td>
</tr>
<tr>
<td>Awake</td>
<td>151 ± 21</td>
<td>158 ± 16</td>
<td>143 ± 14</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>30.1 ± 6.4</td>
<td>26.4 ± 4.5$^b$</td>
<td>31.7 ± 4.2$^b$</td>
</tr>
<tr>
<td>EELV, ml/kg</td>
<td>69.4 ± 7.6</td>
<td>66.5 ± 3.9</td>
<td>71.6 ± 3.8$^b$</td>
</tr>
<tr>
<td>Alveolar PO$_2$, mmHg during rebreathing</td>
<td>119 ± 6</td>
<td>117 ± 9</td>
<td>116 ± 5</td>
</tr>
<tr>
<td>Breathing 21% O$_2$</td>
<td>590 ± 31</td>
<td>592 ± 27</td>
<td>611 ± 35</td>
</tr>
<tr>
<td>Breathing 90% O$_2$</td>
<td>179 ± 56</td>
<td>149 ± 8</td>
<td>140 ± 21</td>
</tr>
<tr>
<td>Pulmonary blood flow, ml (min·kg$^{-1}$)</td>
<td>0.63 ± 0.14</td>
<td>0.55 ± 0.08</td>
<td>0.69 ± 0.12$^b$</td>
</tr>
<tr>
<td>DL$_{CO}$, ml (min·mmHg·kg$^{-1}$)</td>
<td>1.62 ± 0.35</td>
<td>1.46 ± 0.22</td>
<td>1.77 ± 0.28$^b$</td>
</tr>
<tr>
<td>DL$_{NO}$, ml (min·mmHg·kg$^{-1}$)</td>
<td>0.97 ± 0.25</td>
<td>0.79 ± 0.17</td>
<td>0.85 ± 0.19</td>
</tr>
<tr>
<td>DM$_{CO}$, ml (min·mmHg·kg$^{-1}$)</td>
<td>3.14 ± 1.73</td>
<td>2.91 ± 0.68</td>
<td>3.51 ± 0.42</td>
</tr>
<tr>
<td>Vc, ml/kg</td>
<td>5.29 ± 1.87</td>
<td>4.06 ± 1.22</td>
<td>3.57 ± 1.26$^b$</td>
</tr>
</tbody>
</table>

Means ± SD. Inflation volume, 30 ml/kg above end-expiratory lung volume (EELV). EELV, end-inspiratory lung volume. DL$_{CO}$ was expressed at alveolar PO$_2$ = 120 mmHg and hemoglobin = 14.6 g/dl. $^aP < 0.05$ vs. time in both groups by repeated-measures ANOVA. Fisher’s post hoc: $^bP < 0.05$ vs. Placebo Pre-PNX, $^cP < 0.05$ vs. Placebo 4 mo post-PNX, $^dP < 0.05$ vs. RA Pre-PNX, $^eP < 0.05$ vs. RA 4 mo post-PNX. There were no significant differences between Placebo and RA groups at any time point.
shown in Table 4. In RA-treated animals compared with placebo, volume densities of alveolar air spaces were significantly lower while the volume density of septum per unit lung volume was 27% higher. The volume density of tissue per unit lung volume was 29% higher due to increases in collagen fibers, cells, and matrix. Volume density of capillary blood was 27% higher. Volume densities of type 1 epithelium as well as the interstitial collagen fibers, cells, and matrix were 30 to 37% higher. Volume density of endothelium was elevated 30%. Alveolar and capillary surface densities were ~25% higher.

Absolute volumes and surface areas of the left lung are shown in Table 5. In RA-treated group compared with placebo, volumes of the distal air spaces were not significantly different between groups. The volumes and surface areas of alveolar septal components show a trend of higher values in the RA-treated group, but only the increase in the absolute volume of interstitium, including collagen fibers and cells/matrix, reached statistical significance. However, when the septal quantities were expressed and analyzed as ratios to the respective mean control (placebo) values, all ratios in the RA group were significantly elevated; the greatest increase was in the volume of interstitium (1.32) (Fig. 6). Because the increase in barrier resistance offset the increases in capillary blood volume and surface areas in the RA-treated group, morphometric estimates of the conductance of tissue-plasma barrier (DbO₂) and overall conductance of the lung (DLo₂) were unchanged compared with the placebo group (Table 5).

**Discussion**

Summary of results. This study demonstrates that RA supplementation for 4 mo following right PNX permanently altered alveolar septal morphology when studied 16 mo after discontinuation of supplementation. Resting physiological

Table 2. Physiological measurements during exercise

<table>
<thead>
<tr>
<th></th>
<th>Light Exercise</th>
<th>Peak Exercise</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Retinoic Acid</td>
</tr>
<tr>
<td>O₂ uptake, ml/(min·kg)⁻¹</td>
<td>42.5 ± 6.5</td>
<td>48.4 ± 8.0</td>
</tr>
<tr>
<td>CO₂ production, ml/(min·kg)⁻¹</td>
<td>39.1 ± 6.5</td>
<td>42.1 ± 7.4</td>
</tr>
<tr>
<td>Ventilation, liters/(min·kg)⁻¹</td>
<td>3.01 ± 1.01</td>
<td>2.75 ± 0.68</td>
</tr>
<tr>
<td>Tidal volume, ml·kg⁻¹</td>
<td>38.1 ± 8.3</td>
<td>45.8 ± 4.2*</td>
</tr>
<tr>
<td>Respiratory rate, breath·min⁻¹</td>
<td>86.8 ± 45.7</td>
<td>61.6 ± 18.7</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>228 ± 30</td>
<td>223 ± 20</td>
</tr>
<tr>
<td>EELV, ml·kg⁻¹</td>
<td>74.0 ± 6.1</td>
<td>72.7 ± 8.4</td>
</tr>
<tr>
<td>EILV, ml·kg⁻¹</td>
<td>127.6 ± 10.8</td>
<td>130.1 ± 12.4</td>
</tr>
<tr>
<td>Alveolar PO₂ during rebreathing, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breathing 21% O₂</td>
<td>125.9 ± 16.6</td>
<td>114.0 ± 6.1</td>
</tr>
<tr>
<td>Breathing 90% O₂</td>
<td>588.4 ± 11.8</td>
<td>606.5 ± 14.0*</td>
</tr>
<tr>
<td>Pulmonary blood flow, ml/(min·kg)⁻¹</td>
<td>302 ± 7</td>
<td>278 ± 44</td>
</tr>
<tr>
<td>DLCO, ml/(min·mmHg·kg)⁻¹</td>
<td>0.84 ± 0.13</td>
<td>0.99 ± 0.18</td>
</tr>
<tr>
<td>DMCO, ml/(min·mmHg·kg)⁻¹</td>
<td>1.20 ± 0.16</td>
<td>1.67 ± 0.59</td>
</tr>
<tr>
<td>Vc, ml·kg⁻¹</td>
<td>4.07 ± 0.83</td>
<td>4.07 ± 1.1</td>
</tr>
<tr>
<td>Septal tissue volume, ml·kg⁻¹</td>
<td>10.1 ± 2.7</td>
<td>7.6 ± 0.9</td>
</tr>
</tbody>
</table>

Mean ± SD. Light exercise was performed at 7 mph, 0% grade. DLCO was expressed at alveolar PO₂ = 120 mmHg and hemoglobin = 14.6 g/dl. *P ≤ 0.05 RA versus Placebo by unpaired t-test.
has been reported to recruit bone marrow cells to the lung (12),
animals (15). Exogenous RA treatment in murine emphysema
but total alveolar surface area was no greater than in control
of alveolar air spaces may appear smaller and more numerous
have been controversial (5). In RA-treated rat lungs the profiles
pretation of RA-induced enhancement of alveolar septation
factors (1). For technical reasons the measurement and inter-
tion of elastin, and increases the expression of vascular growth
In preterm lambs, vitamin A supplementation partially in-
show impaired postnatal alveolar formation (27) associated
the fetal and adult lung; mice bearing deletions of RA receptors
is expressed in
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DLCO expressed under standard conditions continued to im-
and basal resting pulmonary blood flow declined be-
tween 4 and 16 mo post-PNX in both RA and placebo groups,
consistent with ongoing post-PNX adaptation. Compared with
post-PNX placebo controls, the RA-treated animals showed
prominent increases in the volumes of interstitial fibers,
cells, and matrix with modest relative increases in the volumes
of other septal tissue and blood components; 2) thickened
alveolar capillary basal lamina and the air-blood diffusion
barrier; 3) a high prevalence of septal double capillary profiles;
4) modest relative increases in alveolar-capillary surface areas;
and 5) no significant differences in lung mechanics, aerobic
capacity, or cardiopulmonary function at rest or exercise. Thus
RA supplementation significantly altered long-term alveolar-
capillary ultrastructure following right PNX but failed to im-
prove overall lung function.

Effect of RA on alveolar structure. Retinoids bind to nuclear
retinoic acid receptors (RAR-α, -β, and -γ) and retinoid X
receptors to modulate the transcription of nearly 200 genes and
a wide spectrum of biological actions. RAR-β is expressed in
the fetal and adult lung; mice bearing deletions of RA receptors
show impaired postnatal alveolar formation (27) associated
with reduced tropoelastin mRNA expression in lung fibroblasts
and in whole lung elastin content associated with reduced
alveolar surface area and apparently larger air spaces (17, 27).
In preterm lambs, vitamin A supplementation partially in-
creases secondary alveolar septation and alveolar capillary
growth, reduces the expression of tropoelastin and the deposi-
tion of elastin, and increases the expression of vascular growth
factors (1). For technical reasons the measurement and inter-
pretation of RA-induced enhancement of alveolar septation
have been controversial (5). In RA-treated rat lungs the profiles
of alveolar air spaces may appear smaller and more numerous
but total alveolar surface area was no greater than in control
animals (15). Exogenous RA treatment in murine emphysema
has been reported to recruit bone marrow cells to the lung (12),
although the nature and biological role of these cells remain
undefined.

The effects of RA on connective tissue elements are
prominent and well documented, including increased elastin
gene expression in rat lung fibroblasts and fetal explants
(18), and in premature ventilated baboons but without alter-
ing the expression of angiogenesis genes (21). Several
investigators have shown that RA promotes interstitial con-
nective tissue deposition, including selective enhancement
of collagen production in vitro (4) and lung collagen and
elastin content in vivo (17, 33, 34). RA protects against
bleomycin-induced pulmonary fibrosis in mice (28) and
elastase-induced emphysema in rats (16). In newborn rats
exposed to hyperoxia with or without dexamethasone, RA

treatment improves survival but does not prevent the im-
pairment of septation or improve septal formation. These
studies also reported that RA treatment increases alveolar
collagen content likely due to post-transcriptional regulatory
mechanisms since procollagen mRNA level is not increased (33, 34).

In our study, morphological increases in the volume of
collagen fibers and in capillary basal lamina thickness are
also consistent with RA-enhanced collagen production; the
magnitude of volume increase in interstitial elements was
greater at 16 mo post-PNX than at 4 mo (38), consistent
with sustained RA effects beyond the period of supplementa-
tion. RA-treated animals exhibited a high prevalence of
double septal capillary profiles, which are seen more com-
monly in growing or immature lungs while the normal
alveolar profile in adult dog lungs consists of mostly single
capillary profiles. The increase in double capillaries is
consistent with attempted neocapillary formation, perhaps
via the process of intussusception whereby a tissue pillar
grows into and eventually transects an existing capillary into
two segments (14). Presumably, subsequent lengthening and
remodeling of the tissue pillar and capillary walls would
separate these segments into two single capillaries with a net
gain in gas exchange surfaces. For example, in the placebo
group the prevalence of double capillaries was mildly ele-
vated at 4 mo post-PNX (3.6%) but decreased to 2.8% at 20
mo post-PNX and was no longer different from normal
(Sham PNX, 2.6%). In RA-treated lungs, partial alveolar-
capillary remodeling also occurred; the initially elevated
arithmetic mean septal thickness (5.0 µm) decreased to the
control (placebo) level (4.1 µm). The initially increased
double capillaries at 4 mo post-PNX (6.9%) (38) decreased
to 5.1% at 20 mo post-PNX but remained higher at both time
points than the corresponding control (placebo) levels (3.6
and 2.8%). These results suggest that additional factors
besides RA are required for the remodeling of a transected
capillary into two single alveolar capillaries.

Effect of RA on lung function. Because RA supplemen-
tation caused thickening of the capillary basal lamina and
blood-gas diffusion barrier, which overshadowed the mod-
est changes in capillary blood volume and gas exchange
surface areas, the net effect on diffusive gas conductance
across the lung was negligible when estimated either by the
morphometric model or by physiological measurement at a
given pulmonary blood flow. Static lung compliance
improved slightly between 4 and 16 mo following PNX re-
gardless of RA supplementation. We previously found a
volume-dependent lowering of DLNO but not DLCO when measured under anesthesia in animals treated with RA after right PNX; the finding was interpreted as indirect evidence supporting non-uniform distribution of ventilation at low lung volumes (3). This effect was no longer evident at 16 mo post-PNX. These results extend our previous studies of shorter duration in canine post-PNX remaining lungs (3) and studies by others in rodent lungs with elastase-induced emphysema (32) that demonstrate a lack of functional benefit from exogenous RA supplementation. Only one study reported that RA treatment partially and transiently reversed the trend of deteriorating lung mechanics and aerosol-derived measure of non-uniform ventilation in a canine model of papain-induced emphysema (22); however, in that study a placebo treatment group was not included and gas exchange was not assessed.

**Time course of response to RA post-PNX.** Comparing our present data at 20 mo post-PNX (16 mo following RA treatment) to previous data in adult dogs studied by the same methods 4 mo post-PNX (immediately at the end of RA treatment) (38), there was evidence of continued compensatory structural augmentation in the remaining lung between 4 and 20 mo post-PNX in both treatment groups.

During this interval in the placebo group, total epithelial and endothelial cell volumes and alveolar surface area increased ∼23, 44, and 20%, respectively. The corresponding increases in the RA group were 45, 51, and 59%, respectively. Average capillary blood volume further increased by ∼20% in both groups; average capillary surface area further increased 66% and 45% in the placebo and RA groups.

![Figure 4](image)

**Fig. 4.** Representative light and electron micrographs show the smaller acinar air spaces (A, bar = 100 μm), prominent septal interstitial components (B, bar = 2 μm), and thickened alveolar-capillary basal lamina (C, bar = 500 nm) in RA-treated lungs compared with placebo-treated control lungs and an example of double septal capillaries (D, bar = 2 μm).

**Table 3. Morphometric data**

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Retinoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Terminal body weight, kg</td>
<td>23.9 ± 2.5</td>
<td>23.1 ± 2.0</td>
</tr>
<tr>
<td>Total lung volume, ml·kg⁻¹</td>
<td>73.6 ± 6.2</td>
<td>68.5 ± 3.5</td>
</tr>
<tr>
<td>Intact (immersion method)</td>
<td>60.9 ± 5.2</td>
<td>59.4 ± 5.6</td>
</tr>
<tr>
<td>Sectioned (Cavalieri method)</td>
<td>51.0 ± 0.7</td>
<td>49.3 ± 1.3*</td>
</tr>
<tr>
<td>Morphometric capillary hematocrit, %</td>
<td>3.96 ± 0.17</td>
<td>4.06 ± 0.16</td>
</tr>
<tr>
<td>Arithmetic mean thickness of septum, μm</td>
<td>107 ± 0.02</td>
<td>137 ± 0.06*</td>
</tr>
<tr>
<td>Harmonic mean thickness of blood–gas barrier (r̃), μm</td>
<td>1.09 ± 0.02</td>
<td>1.34 ± 0.06*</td>
</tr>
<tr>
<td>Capillary basal lamina thickness, nm</td>
<td>2.8 ± 0.3</td>
<td>5.1 ± 0.5*</td>
</tr>
<tr>
<td>Double capillary profiles, % of total profiles</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>

Mean ± SD. *P ≤ 0.05 versus placebo.
Table 4. Volume and surface densities of septal structures

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Retinoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume density per unit lung volume</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coarse parenchyma to lung</td>
<td>0.9195 ± 0.0010</td>
<td>0.9054 ± 0.0038</td>
</tr>
<tr>
<td>Fine parenchyma to lung</td>
<td>0.8767 ± 0.0107</td>
<td>0.8658 ± 0.0099</td>
</tr>
<tr>
<td>Alveolar sac</td>
<td>0.6885 ± 0.0209</td>
<td>0.6577 ± 0.0110*</td>
</tr>
<tr>
<td>Alveolar duct</td>
<td>0.0745 ± 0.0076</td>
<td>0.0673 ± 0.0048*</td>
</tr>
<tr>
<td>Respiratory bronchioles</td>
<td>0.0371 ± 0.0060</td>
<td>0.0430 ± 0.0049</td>
</tr>
<tr>
<td>Septum to lung</td>
<td>0.0766 ± 0.0271</td>
<td>0.0978 ± 0.0060*</td>
</tr>
<tr>
<td>Surface density per unit lung volume</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total epithelium</td>
<td>0.0132 ± 0.0049</td>
<td>0.0161 ± 0.0009</td>
</tr>
<tr>
<td>Type 1 epithelium</td>
<td>0.0084 ± 0.0029</td>
<td>0.0109 ± 0.0010*</td>
</tr>
<tr>
<td>Type 2 epithelium</td>
<td>0.0048 ± 0.0020</td>
<td>0.0052 ± 0.0002</td>
</tr>
<tr>
<td>Interstitium</td>
<td>0.0128 ± 0.0045</td>
<td>0.0175 ± 0.0012*</td>
</tr>
<tr>
<td>Collagen fibers</td>
<td>0.0110 ± 0.0038</td>
<td>0.0148 ± 0.0012*</td>
</tr>
<tr>
<td>Cells and matrix</td>
<td>0.0018 ± 0.0007</td>
<td>0.0027 ± 0.0002*</td>
</tr>
<tr>
<td>Endothelium</td>
<td>0.0094 ± 0.0035</td>
<td>0.0120 ± 0.0012</td>
</tr>
<tr>
<td>Septal tissue</td>
<td>0.0354 ± 0.0128</td>
<td>0.0456 ± 0.0301*</td>
</tr>
<tr>
<td>Capillary blood</td>
<td>0.0411 ± 0.0148</td>
<td>0.0522 ± 0.0333*</td>
</tr>
</tbody>
</table>

Table 5. Absolute volumes, surface areas and morphometric diffusing capacities

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Retinoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute volume, ml·kg⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coarse parenchyma</td>
<td>55.45 ± 5.29</td>
<td>53.74 ± 4.91</td>
</tr>
<tr>
<td>Fine parenchyma</td>
<td>53.45 ± 5.02</td>
<td>51.39 ± 4.76</td>
</tr>
<tr>
<td>Alveolar sac</td>
<td>42.00 ± 4.51</td>
<td>39.04 ± 3.66</td>
</tr>
<tr>
<td>Alveolar duct</td>
<td>4.53 ± 0.42</td>
<td>3.99 ± 0.49</td>
</tr>
<tr>
<td>Respiratory bronchioles</td>
<td>2.26 ± 0.35</td>
<td>2.55 ± 0.39</td>
</tr>
<tr>
<td>Septum</td>
<td>4.67 ± 1.68</td>
<td>5.80 ± 0.61</td>
</tr>
<tr>
<td>Total epithelium</td>
<td>0.81 ± 0.32</td>
<td>0.96 ± 0.08</td>
</tr>
<tr>
<td>Type 1 epithelium</td>
<td>0.52 ± 0.19</td>
<td>0.65 ± 0.06</td>
</tr>
<tr>
<td>Type 2 epithelium</td>
<td>0.29 ± 0.13</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td>Interstitium</td>
<td>0.79 ± 0.29</td>
<td>1.04 ± 0.09*</td>
</tr>
<tr>
<td>Collagen fibers</td>
<td>0.67 ± 0.25</td>
<td>0.88 ± 0.08*</td>
</tr>
<tr>
<td>Cells and matrix</td>
<td>0.11 ± 0.05</td>
<td>0.16 ± 0.02*</td>
</tr>
<tr>
<td>Endothelium</td>
<td>0.57 ± 0.22</td>
<td>0.71 ± 0.08</td>
</tr>
<tr>
<td>Septal tissue</td>
<td>2.17 ± 0.82</td>
<td>2.70 ± 0.25</td>
</tr>
<tr>
<td>Capillary blood</td>
<td>2.50 ± 0.89</td>
<td>3.10 ± 0.37</td>
</tr>
<tr>
<td>Absolute surface area, m²·kg⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alveolar surface</td>
<td>2.38 ± 0.90</td>
<td>2.87 ± 0.35</td>
</tr>
<tr>
<td>Capillary surface</td>
<td>1.98 ± 0.72</td>
<td>2.40 ± 0.27</td>
</tr>
<tr>
<td>Diffusing capacity for oxygen, ml·min·mmHg·kg⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capillary blood (DcO₂)</td>
<td>8.28 ± 2.94</td>
<td>10.45 ± 1.25</td>
</tr>
<tr>
<td>Tissue-plasma barrier (DbO₂)</td>
<td>6.59 ± 2.36</td>
<td>6.49 ± 0.63</td>
</tr>
<tr>
<td>Lung (DcO₂)</td>
<td>3.66 ± 1.29</td>
<td>3.99 ± 0.40</td>
</tr>
</tbody>
</table>

Mean ± SD. RA, retinoic acid. *P ≤ 0.05 versus placebo.

creased (to 1.34 μm), reflecting both the progressive basal lamina thickening and the increased volume of interstitium (44%). In contrast, in the placebo group these parameters changed minimally during the same period. Thus, while exogenous RA treatment affected various septal components to some extent, beyond the immediate period of administration its major persistent effect was on connective tissue deposition.

Conclusions. Post-PNX RA supplementation stimulated alveolar tissue and capillary growth, neocapillary formation, persistent connective tissue, fiber and matrix deposition, as well as thickening of capillary basal lamina, leading to significant long-term structural alterations in the remaining lung. Some of the anticipated remodeling events that would have augmented diffusive gas exchange occurred in both RA- and placebo-treated lungs, e.g., continued increase in gas exchange surface areas and transition of double capillaries into single capillaries. However, transition of double capillaries into single capillaries was incomplete in RA-treated lungs. In addition, continued deposition of interstitial and connective tissue components occurred only in RA-treated lungs beyond the period of RA supplementation.
As a result, conductance of the alveolar-capillary diffusion barrier and overall lung function were not enhanced by RA supplementation. These results illustrate the importance of appropriate induction of acinar structural remodeling in addition to the enhancement of cellular proliferation and/or hypertrophy in any attempt to manipulate post-PNX lung growth and compensation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors. The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the National Heart, Lung, and Blood Institute or of the National Institutes of Health.

REFERENCES


