Retinoic acid attenuates O₂-induced inhibition of lung septation

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Veness-Meehan, Kathleen A., Richard A. Pierce, Billie M. Moats-Staats and Alan D. Stiles. Retinoic acid attenuates O₂-induced inhibition of lung septation. Am J Physiol Lung Cell Mol Physiol 283: L971–L980, 2002. First published July 12, 2002; 10.1152/ajplung.00266.2001.—Exposure of the newborn lung to hyperoxia is associated with impaired alveolar development. In newborn rats exposed to hyperoxia and studied at day 14 of life, retinoic acid (RA) treatment improved survival and increased lung collagen but did not improve alveolar development. To determine whether RA treatment during exposure to hyperoxia results in late improvement in alveolarization, we treated newborn rats with RA and hyperoxia from day 3 to day 14 and then weaned O₂ to room air by day 20, and studied the animals on day 42. O₂-exposed animals had larger mean lung volumes, larger alveoli, and decreased gas-exchange tissue relative to air-exposed animals, whereas RA-treated O₂-exposed animals were not statistically different from air-exposed controls. Relative to control animals, elastin staining at day 14 was decreased in hyperoxia-exposed lung independent of RA treatment, and, at day 42, elastin staining was similar in all treatment groups. At day 14, elastin gene expression was similar in all treatment groups, whereas at day 42 lung previously exposed to hyperoxia showed increased elastin signal independent of RA treatment. These results indicate that RA treatment during hyperoxia exposure promotes septal formation without evidence of effects on elastin gene expression after 4 wk of recovery.

alveolar development; bronchopulmonary dysplasia; elastin; hyperoxia

BRONCHOPULMONARY DYSPLASIA (BPD) is the most common cause of long-term disability in premature infants. The pathogenesis of BPD is linked to structural lung immaturity and an arrest of development during the transition from a saccular to alveolar morphology. Little is known about the normal regulation of alveolarization, but exposure of the immature human lung to oxygen and positive pressure ventilation impairs alveolarization, resulting in fewer and larger alveoli (24, 25, 27, 37). Exposure to hyperoxia or to glucocorticoids during critical periods of development inhibits second-
not observe a retinoic acid-induced increase in lung septation in animals studied immediately after the period of hyperoxia (51). Whether the potential for lung septation after hyperoxic exposure is maintained by retinoic acid supplementation is unknown. We hypothesized that retinoic acid treatment of newborn rats during hyperoxic exposure would result in additional lung septation and alveolar formation after the period of hyperoxia. Furthermore, because retinoids regulate lung elastin expression, we hypothesized that retinoid treatment during exposure to hyperoxia would be accompanied by changes in alveolar elastin gene expression and elastic fiber deposition.

**METHODS AND MATERIALS**

**Animals and treatments.** Newborn Sprague-Dawley rats were exposed to ≥90% O₂ or room air from postnatal day 3 through day 13, as previously described (51). Beginning on day 3 (day of birth = postnatal day 0) and daily through day 13, animals in each litter were also injected intraperitoneally with diluent (cottonseed oil) or all-trans-retinoic acid in diluent (500 mg/kg; Sigma, St. Louis, MO). Hyperoxia-exposed animals were maintained in enclosed Plexiglas chambers with continuous flow O₂ at 4 l/min. O₂ concentration was monitored twice daily, humidity was maintained at >80%, and CO₂ was removed by soda lime absorption. Chambers were opened one time daily for injections, cage cleaning, and to rotate nursing mothers between litters in room air and hyperoxia. Hyperoxia-exposed pups were in ambient room air for <10 min/day. Room air controls were raised in the same room as O₂-exposed animals and maintained under normal vivarium conditions. The following four treatment groups were studied: 1) diluent/room air, 2) retinoic acid/room air, 3) diluent/O₂, and 4) retinoic acid/O₂.

At day 14, a subset of animals from each treatment group was killed by intraperitoneal injection of pentobarbital sodium (200 mg/kg). For the remaining animals, O₂ was weaned progressively, and the O₂-exposed animals were returned to room air on day 20. Those animals were allowed to recover in room air until day 42 and then were killed with the remaining room air-exposed animals.

The present study protocol was reviewed and approved by the University of North Carolina at Chapel Hill Institutional Review Committee for Animal Studies.

**Tissue preparation.** On days 14 and 42, animals from each group were anesthetized by intraperitoneal injection of pentobarbital sodium (200 mg/kg). The heart and lungs were exposed by thoracotomy, the trachea was cannulated, and ice-cold 10% buffered formalin was instilled at a transpulmonary pressure of 20 cmH₂O. After 15 min, the trachea was ligated, the lungs and heart were removed en bloc and immersed in 10% buffered formalin, and fixation continued overnight at 4°C. After overnight immersion in formalin, the heart and great vessels were removed, and the lungs were equilibrated overnight in PBS at 4°C. Lung volume measurements were made in triplicate in PBS using the volume displacement technique (43).

For staining, the lungs were dehydrated in 50 and 70% ethanol, and the right caudal and left lobes of the lung were isolated and placed together in cassettes and then embedded in paraffin. Sections of 2 or 4 μm were cut from the approximate center of the paraffin block, mounted on Superfrost Plus slides (VWR Scientific, West Chester, PA), and baked overnight at 55°C.

**Histological staining and morphometric studies.** For air space measurements, sections 4 μm thick were cut, mounted on slides, and stained with hematoxylin and eosin. Contiguous air spaces within 300 μm of the pleura in each of six nonoverlapping fields (magnification = ×12.5) were measured in lung sections chosen from five animals in each treatment group, as previously described (51). For elastin staining, sections were deparaffinized and hydrated, and then, according to Hart’s method, sections were immersed in 0.25% potassium permanganate solution for 5 min. Slides were then cleared in 5% oxalic acid and soaked in resorcinfuchsinsolution overnight. After being washed in water, sections were counterstained with tartrazine (yellow), dehydrated in ethanol, cleared in xylene, and mounted (47). When choosing fields for analysis, care was taken to avoid underinflated areas and fields containing large vessels and airways. Sections were viewed under bright-field optics using an Olympus BH2 microscope (Olympus, Lake Success, NY). Images intended for morphometric analysis were acquired through a charge-coupled device camera (Panasonic, Secaucus, NJ) digitized via the Image-Pro Plus Image Processing System (Media Cybernetics, Silver Spring, MD), and archived on a desktop personal computer. Area measurements were performed using the program Scion Image version Beta3b (Scion, Frederick, MD).

For volume density analyses, sections 2 μm thick were cut, mounted on slides, and stained with hematoxylin and eosin. Sections were viewed under bright-field optics using an Olympus BH2 microscope equipped with a ×20 lens. Images were acquired and archived as before. Volume densities of the gas-exchange air space and the gas-exchange tissue were estimated by one observer blinded to the treatment group using a counting grid consisting of 79 points/field. The number of points falling on the compartment of interest (gas-exchange air space, gas-exchange tissue) in 10 separate fields per animal (5 animals/treatment group) was counted and divided by the total number of test points. Points falling on conducting airways and vessels were excluded.

**Cell proliferation.** Lung cell proliferation was estimated in day 14 lungs using immunohistochemical staining for phosphorylated histone H3 (21, 48). Tissue sections 4 μm thick from five animals in each treatment group were mounted on Superfrost Plus (Fisher Scientific, Pittsburgh, PA) slides, baked at 55°C, and stored at room temperature until processed. The lung sections were deparaffinized, hydrated in a descending ethanol series, and washed in Tris-buffered saline. Endogenous peroxidases were inactivated in 60% methanol and 3% H₂O₂, and the sections were blocked overnight in 1–3% serum. Immunohistochemical staining was performed using a rabbit polyclonal IgG for phosphorylated histone H3. Nonimmune rabbit IgG (Sigma) was used as a negative control for immunostaining. Biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibody was used. Antibody-antigen complexes in tissue sections were visualized by the avidin-biotin-horseradish peroxidase method using the Vectastain avidin-biotin complex-horseradish peroxidase kit (Vector Laboratories, Burlingame, CA) and the substrate 3,3'-diaminobenzidine, which forms a brown precipitate. All sections were then very briefly counterstained with hematoxylin. Sections were viewed under bright-field optics using an Olympus BH2 microscope (Olympus) equipped with a ×40 lens. Ten nonoverlapping fields of lung tissue from five animals in each treatment group were acquired through a Spot Jr. digital camera using Spot software (Diagnostics, Sterling Heights, MI) and printed using a color printer. When choosing fields for analysis, care was taken to avoid underinflated areas and
fields containing large vessels and airways. For each animal, between 600 and 1,200 nuclei were counted by one observer blinded to the treatment group. The total number of nuclei and the number of nuclei expressing phosphorylated histone H3 were determined.

In situ hybridization. Tissue sections 4 μm thick were cut from paraffin-embedded specimens, deparaffinized and dehydrated, and then digested with proteinase K for 30 min. The sections were treated with triethanolamine and acetic acid to block nonspecific hybridization. Sections were dehydrated in successive ethanol washes and then baked 30 min at 60°C. 35S-radiolabeled sense or antisense riboprobes specific for rat tropoelastin were synthesized from a cDNA template encoding exons 8–16 of the tropoelastin mRNA, as described previously (40). Sections were hybridized overnight at 55°C and then subjected to a series of washes, including RNase A treatment to reduce nonspecific hybridization. After being washed, sections were dehydrated in successive ethanol washes and then exposed to X-ray film overnight to estimate signal intensity. Slides were then dipped in Kodak photographic emulsion and dried overnight. Specimens were exposed for autoradiography for 2 wk and then developed and counterstained with hematoxylin and eosin.

Statistical methods. Experimental group means and SDs were determined using descriptive statistics. Treatment groups were compared using a one-way ANOVA, and significance was determined using Bonferroni’s correction for multiple comparisons, or for data not normally distributed, the Kruskal-Wallis one-way ANOVA (17). Statistical analyses were performed on a desktop computer using the program SigmaStat, version 1.0 (Jandel Scientific, San Rafael, CA).

RESULTS

Lung volumes. As shown in Table 1, after 4 wk of recovery in room air, the lung volumes of hyperoxia-exposed animals were 50% larger than the lung volumes of control animals of the same age (5,472 ± 1,019 μL vs. 3,691 ± 636 μL, P < 0.05). Hyperoxia-exposed animals treated with retinoic acid during the period of hyperoxic exposure had lung volumes somewhat larger but not significantly different from the lung volumes of control animals and air-exposed animals treated with retinoic acid (4,828 ± 333 vs. 3,691 ± 636 and 3,680 ± 864 μL, respectively).

Lung morphology. As shown in Fig. 1, at 14 and 42 days of age, the lungs of hyperoxia-exposed animals (Fig. 1, C and D) demonstrate thinned alveolar walls and large air spaces compared with air-exposed control (Fig. 1, A and B) and air-exposed retinoic acid-treated (Fig. 1, E and F) animals. At 14 days of age, the lungs of hyperoxia-exposed animals treated with retinoic acid (Fig. 1G) appear no different from the lungs of animals exposed to hyperoxia (Fig. 1C) alone. However, at 42 days of age, the lungs of hyperoxia-exposed animals treated with retinoic acid (Fig. 1H) contain air spaces that are smaller than those of hyperoxia-exposed animals not treated with retinoic acid (Fig. 1D), indicating that retinoic acid ameliorates the effects of hyperoxia on air space development.

Air space size. As shown in Table 2, at 42 days of age the mean air space size was more than twofold greater in nonretinoic acid-treated hyperoxia-exposed animals compared with control (2,387 ± 485 vs. 1,060 ± 102 μm2, P < 0.05). Hyperoxia-exposed animals treated with retinoic acid had mean air spaces that were somewhat larger but not significantly different from the mean air spaces of control animals and room air-exposed animals treated with retinoic acid (1,692 ± 306 vs. 1,060 ± 102 and 1,581 ± 93 μm2, respectively). Consistent with this, the volume density of the gas-exchange air space was increased, whereas the volume density of the gas-exchange tissue was decreased (Table 3) in hyperoxia-exposed animals relative to control. Treatment with retinoic acid resulted in lung air space and tissue volume densities that did not differ significantly from those of the animals exposed to room air. These findings are consistent with retinoic acid treatment attenuating the effects of hyperoxia on alveolar formation in the developing lung.

Cell proliferation. Because differences in rates of cell proliferation at day 14 might contribute to improved alveolarization at day 42, we examined nuclear expression of phosphorylated histone H3, an antigen present in the nuclei of cells preparing to divide, in day 14 lung tissue. As shown in Table 4, relative to room air animals, hyperoxia exposure was associated with an increase in phosphorylated histone H3-positive nuclei (2.28 ± 0.04 10.39 ± 5.38 per 1,000 cells, P < 0.01). Retinoic acid treatment had no effect on phosphorylated histone H3 expression.

Elastic fibers. At day 14, the lungs of room air control animals and room air animals treated with retinoic acid show discreet elastic fibers in alveolar walls and elastin bundles at the tips of secondary crests (Fig. 2). In O2-exposed animals, elastic fibers appear decreased because of a reduction in secondary crests irrespective of retinoic acid treatment. At day 42, dense elastic fibers are localized to the tips of well-developed secondary septae and to bends in alveolar walls of room air controls and room air animals treated previously with retinoic acid. The lungs of day 42 animals exposed to hyperoxia without retinoic acid treatment and then recovered in room air have thick bundles of elastic fibers within alveolar walls unlike the room air controls or day 14 animals treated with hyperoxia. The lungs of animals treated with retinoic acid during hyperoxia exposure and recovered in room air show discreet elastic fibers in alveolar walls and elastic fiber bundles at the tips of secondary septae similar to control animals.

### Table 1. Lung volumes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Lung Volume, μL</th>
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<tbody>
<tr>
<td>Air</td>
<td>5</td>
<td>3,691 ± 636</td>
</tr>
<tr>
<td>Air + retinoic acid</td>
<td>5</td>
<td>3,680 ± 864</td>
</tr>
<tr>
<td>O2</td>
<td>6</td>
<td>5,472 ± 1,019*</td>
</tr>
<tr>
<td>O2 + retinoic acid</td>
<td>5</td>
<td>4,828 ± 333</td>
</tr>
</tbody>
</table>

Table results are mean lung volumes determined by the volume displacement technique ± SD; n, no. of rats. Rats were placed in room air or O2 and injected with diluent or retinoic acid (500 μg/kg) daily from day 3 through day 13. Beginning on day 14 O2 concentration was weaned with a return to room air on day 20. All rats were killed at 42 days of age. *P < 0.05 compared with air control (Kruskal-Wallis one-way ANOVA; Dunn’s method).
Fig. 1. Morphology of representative lung sections from air- and hyperoxia-exposed rats. Shown are photomicrographs from day 14 (A, C, E, and G) and day 42 (B, D, E, and H) animals exposed to room air (A and B), hyperoxia alone (C and D), room air and treated with retinoic acid (E and F), and hyperoxia treated with retinoic acid (G and H). At 14 days of age, the lungs of hyperoxia-exposed animals treated with retinoic acid (G) appear no different from the lungs of hyperoxia-exposed animals not treated with retinoic acid (C). At 42 days of age, the lungs of hyperoxia-exposed animals contain large, thin-walled air spaces (D), whereas the lungs of hyperoxia-exposed animals treated with retinoic acid (H) contain air spaces that, although larger than those in room air controls (B), are significantly smaller than those of hyperoxia-exposed animals not treated with retinoic acid (D). Bar = 50 μm.
Tropoelastin gene expression. In situ hybridization studies of day 14 control lung showed focal signal for tropoelastin mRNA in alveolar septal tips and discrete sites within alveolar walls (Fig. 3). Sections hybridized with control, sense riboprobes showed no specific hybridization (data not shown). This focal pattern of elastin expression is typical of alveologizing lung, and the signal colocalizes with alveolar myofibroblasts. Retinoic acid-treated animals exposed to room air demonstrated similar localization of tropoelastin mRNA expression, with stronger signal corresponding to the numbers of developing septae in a field of view. In hyperoxia-exposed lung at day 14, a focal pattern of expression similar to that of control lung was noted. Retinoic acid treatment combined with hyperoxia exposure at this early time point did not appreciably alter the localization of tropoelastin mRNA expression. At day 42, signal for tropoelastin mRNA was a reduced air-exposed lung relative to day 14 lung. This reduction is expected, since elastin expression is tightly linked to alveolarization, and alveologization is essentially complete at this age in rodents. However, few studies have examined the patterns of tropoelastin expression in the postnatal lung. Tropoelastin mRNA was no longer detectable in cells of most alveolar septal tips in air-exposed rat lung specimens at day 42, although in animals previously treated with retinoic acid some alveolar walls showed more focal tropoelastin gene expression. In lung specimens from rats exposed to hyperoxia and recovered for 4 wk, signal for tropoelastin is diffuse, with focal areas of stronger expression in alveolar walls. Although the overall amount of signal for tropoelastin mRNA appeared somewhat increased relative to control day 42 lung, the pattern of expression was not reminiscent of alveologizing lung. In retinoic acid-treated, hyperoxia-exposed lung specimens, a similar but stronger pattern of positive signal for tropoelastin mRNA was found in alveolar walls at day 42.

**DISCUSSION**

The transition of the structurally immature lung from a saccular morphology with limited gas-exchange area to an architecturally mature lung with a large internal surface area requires extensive subdivision of gas exchange units and thinning of the alveolar walls. The process of subdivision involves growth of secondary septae from primary alveolar walls. In rats and mice, such subdivision begins around postnatal days 3 and 4 and is virtually complete by postnatal day 14 (11). In humans, the process begins around 36 wk of gestation and continues into and perhaps beyond early childhood (18). Although the signals that regulate the transition are poorly understood, it is known that disrupting lung septation during this period will impair alveolar formation and result in a loss of lung internal surface area (2, 30). A variety of agents, including O2, are capable of causing such an “arrest” of alveolarization. In human premature infants, hyperoxia exposure is a significant risk factor for impaired alveolarization and decreased lung function characteristic of BPD (25, 38). In animal models, we and others have demonstrated that exposure to hyperoxia during critical periods of development impairs lung septation, decreases internal surface area, and results in emphysematous changes similar to those found in patients with BPD (2, 41, 51, 53–55). It had been assumed that such hyperoxia-induced changes in lung architecture are permanent and irreversible once the period of normal alveolar development ends. Until recent descriptions of the effects of vitamin A on this process, there was little evidence that therapeutic agents might promote alveolar development beyond the normal period for lung septation.

Vitamin A (retinol) and its more active metabolite, retinoic acid, have long been recognized for their roles in normal lung cell growth and function and in early branching morphogenesis in lung (12, 13). Clinical studies indicate that vitamin A supplementation may be of benefit to extremely low-birth-weight infants in

### Table 2. Mean air space area

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Air space Area, μm²</th>
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<tbody>
<tr>
<td>Air</td>
<td>5</td>
<td>1,060 ± 102</td>
</tr>
<tr>
<td>Air + retinoic acid</td>
<td>6</td>
<td>1,581 ± 93</td>
</tr>
<tr>
<td>O₂</td>
<td>6</td>
<td>2,387 ± 485</td>
</tr>
<tr>
<td>O₂ + retinoic acid</td>
<td>5</td>
<td>1,692 ± 306</td>
</tr>
</tbody>
</table>

Results are mean values ± SD; n, no. of rats. Rats were placed in room air or O₂ and injected with diluent or retinoic acid (500 μg/kg) daily from day 3 through day 13. Beginning on day 14 O₂ concentration was weaned with a return to room air on day 20. All rats were killed at 42 days of age. *P < 0.05 compared with air control (Kruskal-Wallis one-way ANOVA; Dunn’s method).

### Table 3. Volume density (air space vs. tissue)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Air Space</th>
<th>Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>5</td>
<td>0.6980 ± 0.026</td>
<td>0.3020 ± 0.026</td>
</tr>
<tr>
<td>Air + retinoic acid</td>
<td>6</td>
<td>0.7220 ± 0.035</td>
<td>0.2780 ± 0.035</td>
</tr>
<tr>
<td>O₂</td>
<td>6</td>
<td>0.7670 ± 0.041</td>
<td>0.3290 ± 0.044</td>
</tr>
<tr>
<td>O₂ + retinoic acid</td>
<td>5</td>
<td>0.7380 ± 0.039</td>
<td>0.2580 ± 0.034</td>
</tr>
</tbody>
</table>

Results are volume density ± SD; n, no. of rats. Rats were placed in room air or O₂ and injected with diluent or retinoic acid (500 μg/kg) daily from day 3 through day 13. Beginning on day 14 O₂ concentration was weaned with a return to room air on day 20. All rats were killed at 42 days of age. *P < 0.05 compared with air control (one-way ANOVA, corrected for multiple comparisons).

### Table 4. Phosphorylated histone H3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Phosphorylated Histone H3</th>
</tr>
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<tbody>
<tr>
<td>Air</td>
<td>5</td>
<td>2.28 ± 0.04</td>
</tr>
<tr>
<td>Air + retinoic acid</td>
<td>5</td>
<td>3.07 ± 1.95</td>
</tr>
<tr>
<td>O₂</td>
<td>5</td>
<td>10.39 ± 5.38*</td>
</tr>
<tr>
<td>O₂ + retinoic acid</td>
<td>5</td>
<td>9.97 ± 4.62*</td>
</tr>
</tbody>
</table>

Results for no. of cells positive for phosphorylated histone H3/1,000 cells counted ± SD are shown; n, no. of rats. Rats were placed in room air or O₂ and injected with diluent or retinoic acid (500 μg/kg) daily from day 3 through day 13. All rats were killed at 14 days of age. *P < 0.05 compared with air control (one-way ANOVA, corrected for multiple comparisons).
decreasing their risk of chronic lung disease (44, 45, 49). In normal newborn rat pups, retinoic acid treatment promotes alveolar development and increases the number of alveoli, indicating that it might be of benefit in disease states characterized by failed septation (31).

Studies of adult mice with a genetically determined failure of lung septation (33), rats with dexamethasone-induced inhibition of alveolarization (31, 33), and mice with elastase-induced emphysema (1, 32) have also shown that retinoids promote post hoc alveolar formation in the mature lung. This suggests that retinoids have the potential to be effective in treating chronic lung disease in humans.

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Fig. 3. Elastin gene expression in neonatal and adolescent rat lung. Formalin-fixed, paraffin-embedded tissue was sectioned at 4 μm, hybridized in situ for tropoelastin mRNA, and then counterstained. Signal for tropoelastin mRNA is visible as white (silver) grains in the dark-field views. A: day 14 neonatal rat lung exposed to room air. Tropoelastin mRNA expression localizes to specific cells in alveolar septal tips and bends in alveolar walls. B: day 14 neonatal rat lung exposed to room air and treated with retinoic acid. Sites of expression are more abundant than in room air control lungs. C: day 14 neonatal rat lung exposed to ≥90% O₂. Signal for tropoelastin mRNA localizes to tips of sparse alveolar septae. D: day 14 neonatal rat lung exposed to hyperoxia and treated with retinoic acid during hyperoxic exposure. Tropoelastin mRNA expression patterns are similar to lung exposed to hyperoxia alone. E: day 42 rat lung exposed to room air. Tropoelastin gene expression is not detectable above background. F: day 42 rat lung exposed to room air and treated with retinoic acid. Signal is similar to air-exposed control lung with minimally increased focal expression. G: day 42 rat lung after recovery from hyperoxia. Weak signal for tropoelastin mRNA is diffuse with rare localized expression at bends in alveolar walls. H: day 42 lung of rats treated with retinoic acid during exposure to hyperoxia. Diffuse signal and localized areas of expression for tropoelastin mRNA are found at diverse sites in alveolar walls. Bar = 50 μm.
formation. Our study supports the concept that retinoic acid administration to hyperoxia-exposed rat pups results in post hoc improvements in lung septation.

In earlier studies, we demonstrated that retinoic acid treatment of newborn rats during exposure to hyperoxia improved survival and was associated with increased lung collagen. However, increased alveolar formation was not found in those animals studied immediately after the period of exposure to hyperoxia (51). In contrast, our current study shows improved lung septation and air space development after 4 wk of recovery when animals treated with retinoic acid during exposure to hyperoxia are compared with animals exposed to hyperoxia alone. This is consistent with a role for retinoic acid treatment in the preservation of the potential for septation beyond the normal developmental window. These results were obtained even though retinoic acid treatment was discontinued before the recovery period. Although retinoic acid levels were not measured, it is likely that animals treated with retinoic acid had large tissue stores relative to untreated animals. If that were the case, the effect of retinoic acid on promoting septal development could extend beyond the treatment period.

Because septal growth is thought to proceed, in part, through cell division at specific target sites on primary alveolar walls, the percentage of mitotic cells in representative lung sections taken at the end of the hyperoxic exposure but before recovery was also examined. Hyperoxic exposure was associated with an increase in cell proliferation, based on phosphorylated histone H3 nuclear staining, in both retinoic acid-treated and untreated groups, but there was no difference in this cell proliferation index between the two groups.

Retinoic acid has been demonstrated to affect gene expression of >300 gene products (15). Because retinoids are antioxidants (26), they are also thought to protect the lung from damage by O2 free radicals generated through direct O2 exposure and from inflammatory cells, both thought to participate in hyperoxia-induced arrest of lung septation. There is evidence, however, that retinoids may exert their primary influence in lung through effects on genes involved in morphogenesis (5, 12, 13, 28, 39) and in extracellular matrix synthesis (15, 16, 19, 20, 34, 50, 51). One of the extracellular matrix components most critical to the process of lung septation and alveolar formation is elastin, a developmentally regulated gene in lung (7, 56). First expressed near points of airway branching during the pseudoglandular stage of lung development, elastin synthesis is greatest in the newborn lung during alveolar formation. Elastin is concentrated at the tips of developing septae, forming rings that surround the mouths of alveoli, and in bundles in the alveolar walls (36). Although the factors that regulate elastin synthesis during lung development are not known, retinoids are known to upregulate elastin expression in alveolar myofibroblasts in vitro. In addition, retinyl esters accumulate in fetal rat lung before birth, and retinoic acid receptor and binding protein expression in lung peak during the period of most active lung septation (14, 22, 35). Furthermore, mice carrying deletions of retinoic acid receptors demonstrate decreased lung elastin and impaired alveolarization (34), indicating a link between elastin, retinoic acid, and lung alveolar formation. In the work presented here, we have demonstrated decreased lung elastin in hyperoxia-exposed, 14-day-old animals immediately after exposure to hyperoxia, in conjunction with a reduction in secondary crests. In the lungs of recovered animals at day 42, lack of septation is still evident, although thickened bundles of elastic fibers are now evident in alveolar walls. These results are consistent with studies showing decreased elastin (tropoelastin) gene expression (6, 9) and increased elastin degradation (10) in newborn rat lung after exposure to hyperoxia with a rebound in elastin gene expression after return to room air (8). In hyperoxia-exposed animals treated with retinoic acid, alveolar wall elastin is similar to that of hyperoxia-exposed animals not treated with retinoic acid. However, dense elastin bundles are evident at the tips of alveolar crests immediately after exposure to hyperoxia. After recovery, the architecture of hyperoxia-exposed retinoic acid-treated lung is similar to that of animals not exposed to O2, but tropoelastin gene expression is similar to that of animals exposed to hyperoxia alone.

Although the elastin staining assesses the form and amount of accumulated elastin in alveolar walls, in situ hybridization offers a glimpse of tropoelastin gene expression at a specific time point. As anticipated, tropoelastin expression localized primarily to alveolar septal tips in control, air-exposed rat lung at 14 days. As Bruce and co-workers (6) previously noted, exposure to hyperoxia diminished tropoelastin gene expression in alveolar walls. Interestingly, this reduction appears to be not only because of downregulation of tropoelastin gene expression on a per-cell basis but may also involve a reduction in the numbers of tropoelastin-expressing cells in alveolar walls. This finding strengthens the notion that elastin expression is closely linked to alveolarization. In our studies, irrespective of hyperoxia exposure, retinoic acid treatment did not appear to appreciably alter tropoelastin expression patterns at 14 days of age.

At 42 days of age, alveolarization is considered “complete” in rats (3, 4). Therefore, it is not surprising that extensive areas of lung parenchyma showed little detectable signal for tropoelastin mRNA in the room air-exposed lung at 42 days. This process appears to involve “extinguishing” tropoelastin expression in clusters of alveoli emanating from the same alveolar duct. Other areas have reduced but detectable expression of tropoelastin, suggesting that alveolarization may be completed at slightly different times in clusters of alveoli within a single lobe (data not shown).

In lung previously exposed to hyperoxia and treated with retinoic acid, tropoelastin expression is still detectable in alveolar walls at day 42. However, although detectable, return to normoxia did not initiate an alveolar pattern of expression of tropoelastin. Instead, tropoelastin was expressed at low levels at many sites
within extended alveolar walls. The more diffuse elastin expression may indicate that the retinoid-mediated beneficial effect on alveolar septation occurred earlier in the recovery period and continued focal expression of elastin may not be necessary for increased structural complexity.

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