

Retinoids Increase Lung Elastin Expression But Fail to Alter Morphology or Angiogenesis Genes in Premature Ventilated Baboons

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ABSTRACT: Retinoids regulate elastin synthesis by alveolar myofibroblasts and affect angiogenesis pathways, both of which are processes critical for alveolar development. Retinoids accelerate alveolarization in rodents and are now used therapeutically in premature infants at risk of bronchopulmonary dysplasia (BPD). This study examined the effects of retinoid supplementation on alveolar elastin expression and deposition and angiogenesis-related signaling in a primate model of BPD. Premature baboons delivered at 125 d of gestation after maternal steroid treatment were given surfactant and ventilated with minimal supplemental oxygen for 14 d with ($n = 5$) and without ($n = 5$) supplemental vitamin A (5000 U/kg/d) and compared with 140-d unventilated controls. Ventilatory efficiency index (VEI) and oxygenation index (OI) were not statistically different between ventilated treatment groups. Expression of vascular endothelial growth factor A (VEGF-A), fms-related tyrosine kinase 1 (Flt-1), and tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (TIE-1) was repressed by premature delivery and mechanical ventilation and was not altered by retinoid supplementation. Retinoid supplementation did not enhance alveolar angiogenesis. Elastin expression was repressed by premature delivery and extended ventilation, and retinoid supplementation increased elastin expression specifically in alveolar myofibroblasts within alveolar walls. These results suggest that the small decrease in mortality among premature infants receiving retinoid supplementation may not be mediated through enhanced alveolar development. (*Pediatr Res* 61: 703–709, 2007)

Many premature infants develop respiratory distress and often require mechanical ventilation and supplemental oxygen to survive. These interventions can perpetuate injury and ultimately lead to altered lung architecture BPD (1), with failed alveolar development, which is a hallmark of BPD (2). The incidence of BPD remains high in infants of very low birth weight despite advancements such as surfactant therapy.

The findings that retinoid signaling is required for alveolar development in mice and that retinoid treatment can enhance alveolar development in rats (3–5) led to vitamin A supple-

mentation of preterm infants at risk of BPD. Some clinical trials of vitamin A supplementation in premature infants have shown no benefit, and others have found that dose is critical (6–8). Tyson *et al.* (9) found that overall, one additional infant survived without chronic lung disease for every 14–15 infants who received vitamin A supplements. The mechanism by which vitamin A supplementation may reduce BPD or mortality is not well defined, but may be linked to enhanced alveolar development.

The altered alveolar capillary network associated with BPD (10–12) supports the “vascular hypothesis” that failed capillary development is central to the pathogenesis of BPD (13). Retinoid signaling is required for alveolar capillary development. Vitamin A supplementation may decrease the incidence of BPD by stimulating VEGF expression in alveolar epithelial cells (11,14) or through other proangiogenic pathways (15). Retinoid signaling also increases lung endothelial proliferation (16).

Vitamin A supplementation may also stimulate alveolar development by enhancing elastin expression or deposition by alveolar myofibroblasts, which are retinoid responsive and dependent (17,18). The mechanical properties of distensibility and recoil of alveoli, facilitating influx of fresh air and exhalation of carbon dioxide from respiration, are conferred by elastic fibers encircling the openings of alveoli. Mice with genetic ablation of genes required for elastic fiber assembly or elastin expression have failed alveolar development (19).

To test the effects of retinoid supplementation on the incidence of BPD, alveolar capillary development, and alveolar elastic fiber deposition, we used an experimental model of BPD in the baboon (term = 185 d). This model uses antenatal steroid therapy before premature delivery, surfactant therapy, and mechanical ventilation of premature baboons delivered at 125 d of gestation, with supplemental oxygen only as required. Retinoid supplementation stimulated alveolar elastin

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Abbreviations: α -SMA, α -smooth muscle actin; BPD, bronchopulmonary dysplasia; FLT-1, fms-related tyrosine kinase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PECAM-1, platelet/endothelial cell adhesion molecule 1; RPA, ribonuclease protection assay; TIE-1, tyrosine kinase with immunoglobulin-like and EGF-like domains 1; VEI, ventilatory efficiency index

expression but not alveolar angiogenesis, capillary development, or alveolar size in this study.

MATERIALS AND METHODS

Animals. Animal treatments were performed at the Southwest Foundation for Biomedical Research (San Antonio, TX) in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. Fetal baboons are delivered by hysterotomy at 125 ± 2 d or 140 ± 2 d of gestation after antenatal steroid therapy (betamethasone 6 mg at 48 h and 24 h before delivery). This is approximately equivalent to 27 wk of gestation in humans. After delivery, baboons were intubated, treated with surfactant (Survanta at 4 mg/kg), and placed on positive pressure ventilation with tidal volumes of 4–6 mL/kg to maintain P_{CO_2} of 45–55 mm Hg and supplemental O_2 as needed to maintain arterial P_{O_2} at 40–60 mm Hg for 14 d. Proper fluid supplementation, nutrition, and antibiotic support were provided. Ventilatory management, hemodynamic support, pulmonary function testing, and postmortem pressure-volume measurements were conducted as described (20). Five animals received daily retinoid supplementation (5 mg/kg, Aquasol A delivered by intramuscular injection), and five received vehicle injections alone. Gestational control animals included premature baboons delivered at 140 d (5). At the time of killing by intravenous infusion of excess sodium pentobarbital sodium, the lungs were cleared by infusion of phosphate-buffered saline (PBS) in the right heart, and then lung tissue was snap-frozen in liquid nitrogen or inflated with 4% paraformaldehyde at 20 cm pressure for fixation.

Morphometry. Two sections from each of one to two blocks of right upper lung lobe tissue per animal in the study were stained with hematoxylin and eosin. Ten digital images at 200 \times magnification per section were captured using a random starting point and a random number generator, and then analyzed for chord length using NIH Image software (Version 1.63, National Institutes of Health, Bethesda, MD) with a chord length macro (available at <http://rsb.info.nih.gov/nih-image>) (21). Tissue density was calculated by assessing air space and tissue using Image Pro Plus software and the formula: Tissue density = area occupied by tissue/total area (tissue + air space).

Ribonuclease protection assay (RPA). RPA were performed as described previously (11). The hAngio-1 riboprobe template (Pharming, San Diego, CA) was used to generate labeled antisense riboprobes. After electrophoresis, the intensity of each specific message band was measured using a computer-linked PhosphoImager with ImageQuant software (Molecular Dynamics) and normalized to the intensity of the constitutively expressed housekeeping gene L32.

Platelet/endothelial cell adhesion molecule 1 (PECAM-1) immunohistochemistry. PECAM-1 immunohistochemistry was performed with antigen retrieval in 50 mM Tris-HCl, pH 9.5 with microwave treatment (11). The slides were treated with H_2O_2 to block endogenous peroxidase activity and blocked in triethanolamine-buffered saline (TBS) + 3% horse serum. Sections were incubated overnight with primary goat anti-PECAM-1 polyclonal antibody (Santa Cruz, sc-1506) and diluted 1:200 in TBS/1.5% horse serum at 4°C. Biotinylated horse anti-goat IgG diluted 1:200 in TBS/1.5% horse serum was added for 45 min, followed by ABC-AP reagent. Chromogenic substrate was added, and the slides were incubated for 15–20 min. Slides were counterstained with hematoxylin and eosin. The negative control was goat IgG in TBS/1% horse serum.

Hart's elastin staining. Rehydrated peripheral lung tissue sections (5 μ m) were soaked in 0.25% potassium permanganate solution for 5 min, cleared in 5% oxalic acid, and soaked in resorcin-fuchsin solution (Poly Scientific, Bay Shore, NY) overnight. After washing in water, sections were counterstained with tartrazine (yellow), dehydrated through graded ethanol, cleared in xylenes, and mounted.

Staining for α -smooth muscle actin (α -SMA). Immunofluorescent staining for α -SMA of rehydrated sections used overnight incubation at 4°C with the primary antibody (1A4 mouse monoclonal, 1:200 dilution, Sigma Chemical Co., St. Louis, MO) after blocking with 10% normal horse serum. After PBS washes, sections were incubated in the dark with secondary antibody (fluorescein-conjugated goat anti-mouse IgG, 1:250 dilution, 1 h, room temperature; Jackson ImmunoResearch, West Grove, PA). After PBS washes, sections were viewed under fluorescent light for image capture.

Quantification of elastic fiber density and α -SMA staining. A minimum of 10 random lung fields of stained sections per animal at 200 \times magnification were captured with a digital camera and imported into Image Pro Plus software. For quantification of elastic fiber density, the proportion of the area of each image that was brown to black (elastic fibers) and yellow (nonelastic tissue) was determined with Image Pro Plus software (Media Cybernetics, San Diego, CA). For α -SMA staining, the areas of saccular or alveolar lung tissue with each of three nonoverlapping intensity levels of staining for α -SMA were determined. Images were captured with the same exposure to control for

signal intensity. Statistical analysis was performed using the *t* test for independent samples, two-tailed analysis.

Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) for elastin expression. Total RNA isolated from peripheral lung was used to prepare cDNA using Superscript Plus RT and random hexamer priming. The cDNA was then subjected to PCR amplification for elastin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Primers used were 5' gtc ctg gtc ttc ctg gac tt 3' as the ELN forward primer and 5' agg cac tgc tgc tcc ata tt 3' for the ELN reverse primer, yielding a 205-bp product, and 5' tgc acc acc aac tgc tta gc 3' as the GAPDH forward primer and 5' ggc atg gac tgt ggt cat gag 3' for the GAPDH reverse primer, yielding a 104-bp product with a Stratagene MX 3000 instrument. Standard curves were generated, and results using Sybr Green for detection were standardized using reference cDNA for each run.

In situ hybridization. 35 S-labeled antisense or sense riboprobes were generated from a baboon tropoelastin cDNA linearized for *in vitro* transcription (Promega, Madison, WI). Rehydrated sections of peripheral lung were digested with proteinase K, acetylated, and then hybridized with denatured radiolabeled riboprobe (40,000 cpm/ μ L) overnight at 60°C. After stringent washes including digestion with RNase A, slides were exposed for 11 d and then developed and counterstained with hematoxylin and eosin.

RESULTS

Clinical outcomes: study groups and clinical course.

There were no deaths in the control animals ($n = 5$) and one death in the retinoid-treated group ($n = 5$) due to respiratory failure and unresponsive hypotension. The gender distribution, gestational ages, and birth weights in the two treatment groups were similar. Daily fluid intake, daily urine output, and daily weights over the course of the study were similar between groups.

Pulmonary function. Protocols for ventilation and oxygen support were strictly guided by predetermined goals for blood gas values. The oxygenation index (OI) (Fig. 1A) and ventilatory efficiency index (VEI) (Fig. 1B) were similar for both the experimental groups. There was a trend toward reduced lung volumes achieved at the same inspiratory pressures postmortem in the retinoid-treated group (Fig. 1C). Lung compliance, as measured using a VitalTrends machine, was not statistically different between treatment groups (Fig. 1D).

Morphology. The 140-d gestational control lungs exhibit an alveolar appearance as previously described in this model (22) (Fig. 2A). Alveolar walls were relatively thin and had numerous septal projections (Fig. 2B). By comparison, lungs from animals delivered at 125 d and ventilated for 14 d displayed enlarged terminal air spaces with extended walls and a lack of septation in the untreated (Fig. 2C) and retinoid-treated (Fig. 2E) animals. In other regions of the same lungs, thickened hypercellular saccular walls were found in the untreated (Fig. 2D) and retinoid-treated (Fig. 2F) groups. The varied morphology in the ventilated groups was reflected in the greater variance in chord length compared with gestational controls. The mean chord length showed a trend toward increase with ventilation but did not achieve statistical significance. However, when the frequency of classes of chord lengths was assessed between groups, the retinoid-treated lungs showed an increase in the largest sized terminal air spaces compared with gestational controls.

Expression of angiogenesis-related genes. Because retinoid acid increases fetal lung endothelial cell proliferation (23) and may modulate VEGF expression (24), we analyzed mRNA expression of several angiogenesis-related genes by

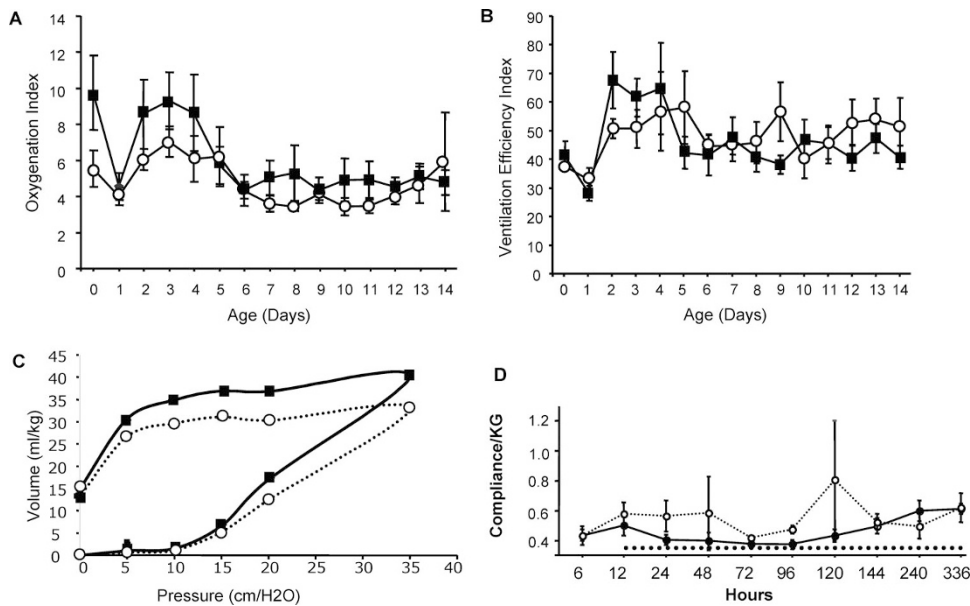


Figure 1. Physiologic effects of retinoid treatment. Shown are values for individual animals. Ventilation only (■). Ventilation with retinoid supplementation (□). (A) $OI = F_{iO_2} \cdot 100 \cdot P_{aw} / P_{aO_2}$. (B) $VEI = P_{aCO_2} \cdot \text{rate} \cdot PIP / 1000$. (C) Postmortem pressure-volume analysis. (D) Dynamic compliance measurements from throughout the course of treatment did not show statistical differences. Control (○); retinoid (●).

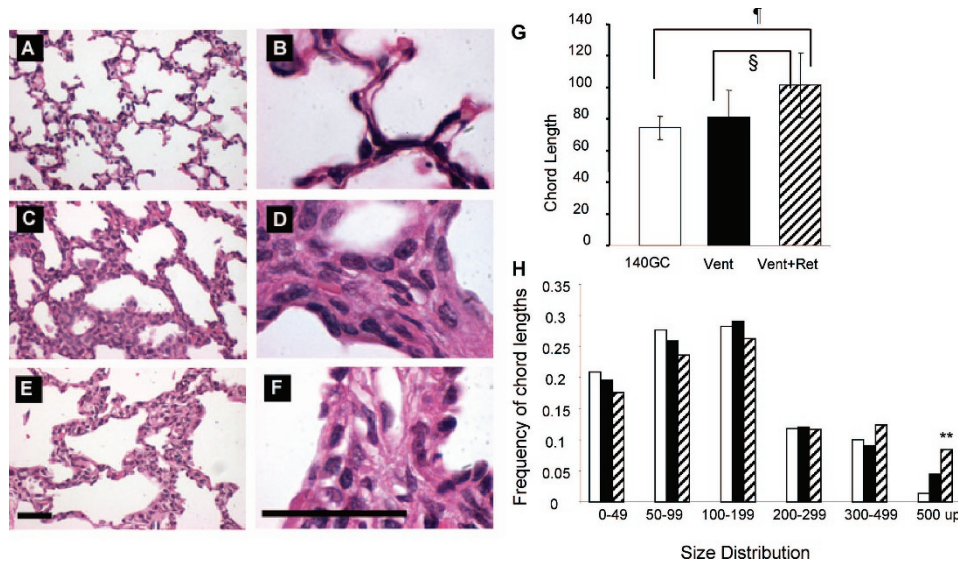


Figure 2. Morphology of premature baboon lung. Hematoxylin and eosin–stained panels at $200\times$ (A, C, E) and $1000\times$ (B, D, F). (A, B) At 140 d, gestational control lungs exhibit alveolar morphology. (C, D) Premature baboons delivered at 125 d and ventilated for 14 d exhibit simplified, enlarged air spaces lacking alveolar wall extensions (C) and thickened saccular walls (D). Lungs from the retinoid supplementation group (E, F) show similar developmental deformities. Bar = $100 \mu\text{M}$. (G, H) 140-d gestational control (open columns), ventilation-only (solid columns), and retinoid-treated (hatched columns) animals.

RPA (Fig. 3). VEGF, FLT-1, and TIE-1 were significantly decreased in both treatment groups compared with the 140-d gestational controls.

PECAM-1 staining. To assess capillary development, sections of peripheral lung were stained for the endothelial marker PECAM-1 (Fig. 4A–C). As reported previously, the 140-d gestational control animals had a well-developed distal capillary network with many capillaries closely apposed to the terminal epithelium of thin inter-air space septa (11). The ventilated untreated animals often had capillaries embedded in the thickened saccular walls and occasional dilated capillaries, as noted previously in this BPD model (11). The retinoid-treated animals had capillary networks that were very similar to the untreated animals.

ELN fiber staining. We assessed elastic fiber staining in the alveolar compartment of the lung, as opposed to whole lung sampling of desmosine, which would include elastin within blood vessels and conducting airways as well as in the pleural surface. Dense elastic fibers localized to the tips of emerging

alveolar septae in 140-d gestational lungs (Fig. 5A), and little elastic fiber staining was found within alveolar walls. In lungs of untreated ventilated animals, dense elastic fibers localized to shortened alveolar extensions and short brushlike elastic fibers were deposited along some saccular walls (Fig. 5B). In lungs of retinoid-treated ventilated animals, elastic fibers often stained more intensely and extended along walls of simplified terminal air spaces (Fig. 5C).

Lung elastin expression in lungs of retinoid-treated, ventilated premature baboons. Because retinoids stimulate expression of elastin in alveolar myofibroblasts, we examined elastin mRNA expression by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) (Fig. 6). Elastin expression was increased 4.7-fold with retinoid treatment.

Localization of increased elastin expression. The localization of elastin mRNA expression was assessed by *in situ* hybridization. Elastin expression was intense in the muscular walls and endothelium of the largest intralobar pulmonary arteries in all treatment groups (Fig. 7A–F). Elastin expression

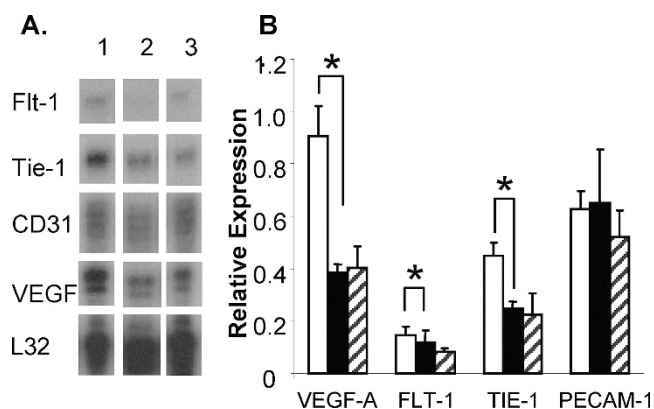


Figure 3. (A) Representative RPA gel for several genes in angiogenesis cluster. (B) Quantification of RPA data showing decreased Flt-1, Tie-1, and VEGF in ventilation-only animals compared with 140-d gestational control animals. There were no differences between the ventilation-only animals and the retinol-treated animals. 140-d gestational control (*open columns*), ventilation-only (*solid columns*), retinol-treated (*hatched columns*) animals. (Four to five animals for each condition. * $p < 0.05$. Data are means \pm SEM.)

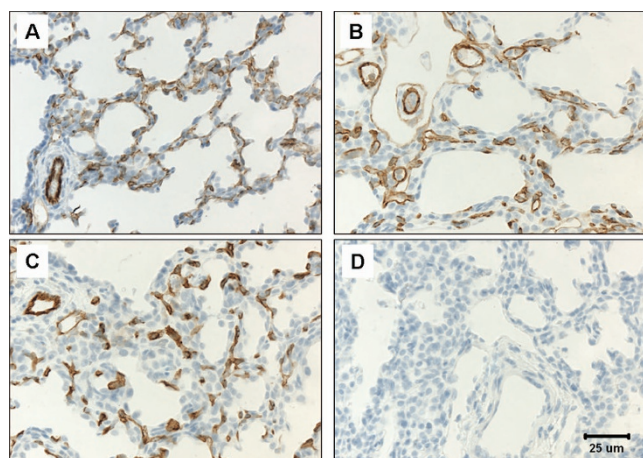


Figure 4. PECAM-1 immunohistochemistry. Representative fields from 140-d gestational control (A), ventilation-only (B), and retinol-treated (C) animals and control antibody (D). The 140-d gestational control animals had an extensive network of distal air space capillaries (brown staining) that were largely subepithelial. The capillary network in the ventilation-only animals appeared fragmented with isolated capillaries in thickened mesenchyme. The retinol-treated animals were similar to the ventilation-only animals.

was detected in adventitial layers in retinoid-treated premature ventilated animals (Fig. 7F) but not in other treatment groups. Large airway smooth muscle exhibited abundant elastin expression in 140-d gestational controls (Fig. 7G and H) was slightly increased in the ventilation-only group (Fig. 7I and J) and the retinoid-treated group (Fig. 7K and L). Signal for elastin mRNA in small airway smooth muscle was modest in all treatment groups (Fig. 7M–R), but vascular elastin expression was high in the same sections. A marked increase in elastin expression was found with retinoid treatment in the alveolar compartment of the lung (Fig. 7S–X). In alveolar walls of control animals, few cells expressed elastin at high levels (Fig. 7S and T). In the ventilated group, intense expression was detected in some cells in the immature alveolar walls and many cells were negative (Fig. 7U and V). In the retinoid-treated group, intense signal for elastin mRNA localized to

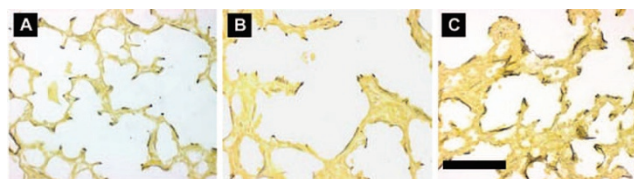


Figure 5. Elastic fibers in alveolar walls. Shown are peripheral lung specimens stained with Hart's elastic fiber stain (brown to black) and counterstained with tartrazine (yellow) at 200 \times magnification. (A) 140-d gestational control specimens exhibit dense and focal elastic fibers localizing primarily to the tips of alveolar walls. (B) Specimens from the ventilation-only group exhibit focal and dense elastic fibers at sites where alveolar walls normally extend and along and within thickened alveolar walls. (C) In lung specimens from the retinoid-treated group, marked thickening of saccular walls was often noted and dense accumulations of elastic fibers were found at sites along saccular walls. Bar = 100 μ M.

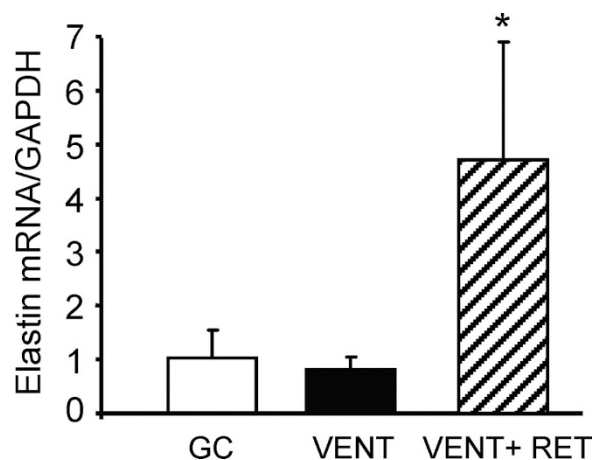


Figure 6. Retinoid treatment increases elastin expression in the ventilated preterm lung. Elastin expression in ventilated retinoid-treated lung specimens (VENT + RET) was increased compared with the ventilation-only group and gestational controls (GC).

individual cells in simplified alveolar walls (Fig. 7W and X). These results are consistent with a marked induction of elastin expression with retinoid treatment, as indicated by the quantitative RT-PCR results.

Changes in α -SMA staining with ventilation and retinoid treatment. The distribution of smooth muscle cells and alveolar myofibroblasts within the walls of terminal air spaces was assessed by staining for α -SMA (Fig. 8). Cells intensely positive for α -SMA were found at bends in alveolar walls and at the tips of alveolar walls in 140-d gestational control lungs (Fig. 8A and B). In ventilated lungs with (Fig. 8E and F) and without retinoid treatment (Fig. 8C and D), intensely staining cells were found at obtuse angles of saccular walls with short, thick extensions. Cells with intermediate and weak but positive intensity staining for α -SMA were abundant in both ventilated treatment groups. Quantitative analysis of these subpopulations showed that cells weakly positive for α -SMA were more abundant in the ventilated treatment groups (Fig. 8G). This population may represent cells transitioning to a smooth muscle-like phenotype in the ventilated premature lung.

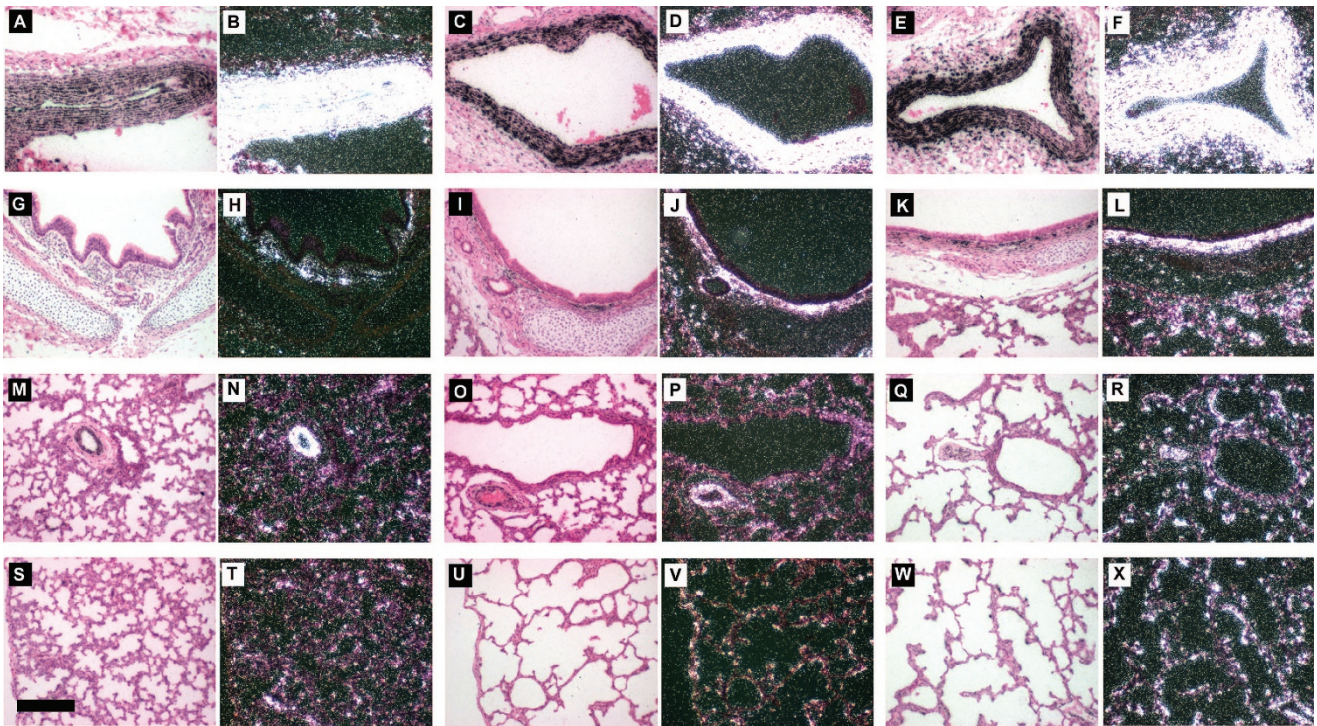


Figure 7. Localization of elastin expression in premature baboon lung. Shown are paired bright- and dark-field views of sections of lung hybridized *in situ* for tropoelastin mRNA. Signal appears as white dots in the dark-field views. (A–F) Intralobar pulmonary arteries. (G–L) Largest airways. (M–R) Paired conducting airways and arteries. (S–X) Peripheral lung sections. (A, B, G, H, M, N, S, T) From the 140-d gestational controls. (C, D, I, J, O, P, U, V) From the ventilation-only group. (E, F, K, L, Q, R, W, X) From the retinoid supplementation group.

DISCUSSION

Angiogenesis and elastic fiber formation are key factors in alveolar development that may be altered in BPD (25), and both these pathways are retinoid sensitive (18,26). Retinoid supplementation is the only treatment to date that increases alveolar number in experimental models (3) and has also been shown to reduce the incidence or severity of BPD in some clinical trials (6). The clinical benefit is a reduction in the combined outcome of oxygen requirement at 36-wk gestational age or reduced mortality for one of every 14 or 15 premature infants receiving vitamin A treatment compared with controls (9). We therefore studied the effects of retinoid supplementation on these parameters and on lung physiology in a primate model of BPD.

Physiologic parameters were not improved by vitamin A supplementation, and the incidence of early death was greater in the vitamin A-supplemented group (1/5) compared with the placebo-treated group. A longer treatment period, altered dose (25), or increasing the number of animals per treatment group may have resulted in a detected benefit of vitamin A supplementation in this model. The results of this study in a primate model are in line with findings that retinoid supplementation may benefit a small subset of infants at risk of BPD.

Retinoid supplementation has yielded variable results in other animal models of BPD (5,27,28), with the most marked effects in rodent models (3,29). It seems unlikely that the antenatal steroid treatment blocked a potential benefit of retinoid supplementation because antenatal steroid therapy was common in studies in which retinoid supplementation showed some benefit in premature infants (9). Furthermore, retinoid

treatment advanced alveolar development in a rodent model in which alveolar maturation was inhibited by postnatal glucocorticoid supplementation (3). These differences suggest that mechanical ventilation of the premature lung promotes signals that are not normalized by retinoid supplementation.

Retinoic acid treatment failed to correct the abnormal microvasculature of terminal air space walls noted previously in this model of BPD (11) or to enhance VEGF expression compared with untreated controls. Reports on the effects of retinoids on angiogenesis vary widely. Retinoic acid increases VEGF expression in lung epithelial cells (14) and stimulates fetal pulmonary microvascular endothelial cell proliferation *in vitro* (23). Retinoids reverse dexamethasone-mediated repression of lung VEGF receptor 2 in newborn mice (30) and restore alveolarization caused by pharmacologic blockade of this receptor (23). In contrast, retinoids arrest angiogenesis in certain tumor subtypes (31) and reduce endothelial cell number and decrease vessel branching in an *in vivo* model of angiogenesis (32). The failure of retinol treatment to prevent abnormal capillaries in this study may be due to dose, timing, or the complex interplay of cell events that are necessary for vascular development.

In contrast to increased elastin expression and fiber deposition in a lamb model of BPD (33), lung elastin mRNA expression was modestly decreased in 125-d gestation baboons delivered prematurely and ventilated 14 d compared with 140-d gestational control animals. This difference may represent what occurs in the “new BPD” found in premature neonates treated with antenatal steroids, surfactant upon birth, and low tidal volume ventilation. Retinoid supplementation of

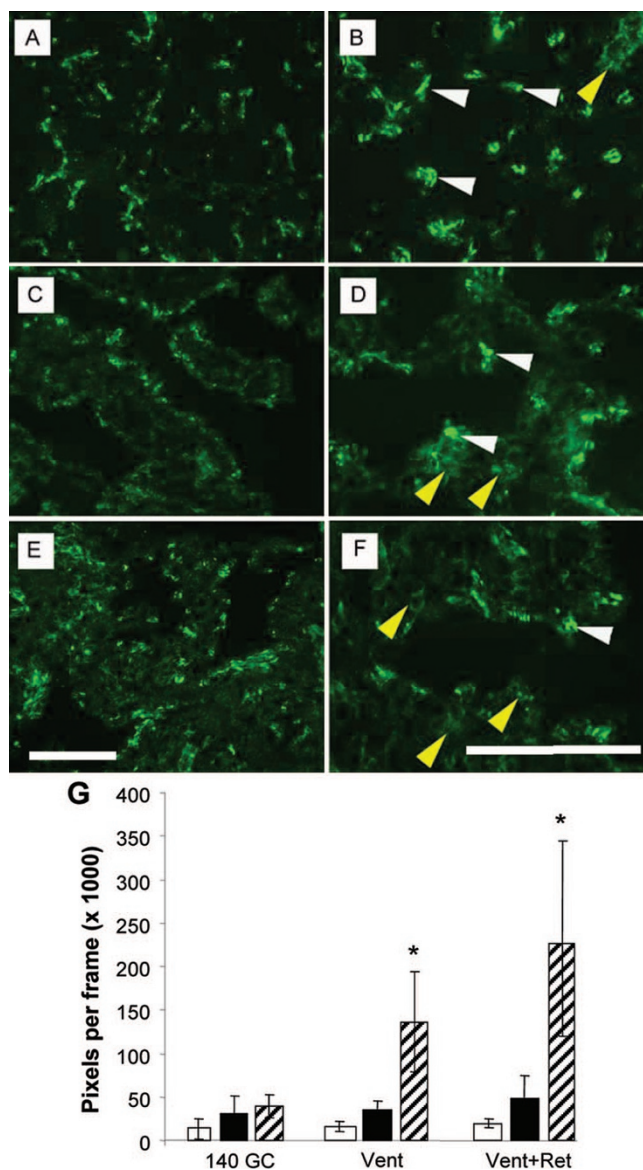


Figure 8. Staining for α -SMA is altered by ventilation of premature lung. Shown are peripheral lung sections stained by immunofluorescence for α -SMA at $\times 200$ (A, C, E) and $\times 400$ (B, D, F). (A, B) 140-d gestational control lung with intense staining (bright green signal, white arrowheads) of small cells in alveolar walls. Few positive cells exhibit intermediate staining (faint green signal) or are large (yellow arrowhead). (C, D) In the ventilation-only group, intensely staining cells do not localize to tips of emerging alveolar septae. More cells exhibit less intense staining and larger size (yellow arrowheads). (E, F) In the retinoid treatment group, cells of both phenotypes are also found. (G) Quantification of area occupied by brightly staining cells (open columns), cells staining with medium intensity (solid columns), and cells slightly positive for α -SMA (hatched columns). * $p < 0.05$.

the ventilated preterm neonate increased lung elastin expression, particularly in cells that are positive for α -SMA in saccular and alveolar walls. This localization is similar to findings in a neonatal rat model of retinoid supplementation during hyperoxia (5). Thibeault *et al.* (34) showed that elastic fiber density is increased in premature infants who died of chronic lung disease after prolonged mechanical ventilation (mean of 29 d), but elastin expression was not quantified or localized in that study.

Mechanical ventilation of the premature lung resulted in a larger number of cells expressing α -SMA staining in the walls of terminal air spaces. In ventilated lungs, cells of larger size staining intensely for α -SMA were found in alveolar walls of ventilated lungs along with cells with intermediate staining intensity. This suggests that there is recruitment of other cell types to a myofibroblast-like phenotype in the ventilated premature lung. The abnormal distribution of elastic fibers in the BPD lung may result from expression and synthesis of elastin by cells with this intermediate phenotype.

An important finding of this study is that altering alveolar elastin expression through treatment with retinoids did not result in improved alveolar development. Positively affecting one system important in alveolar development, without improving angiogenesis, may fail to result in improved alveolar development.

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