Type II epithelial cells are critical target for hyperoxia-mediated impairment of postnatal lung development

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Yee, Min, Peter F. Vitiello, Jason M. Roper, Rhonda J. Staversky, Terry W. Wright, Sharon A. McGrath-Morrow, William M. Maniscalco, Jacob N. Finkelstein, and Michael A. O’Reilly. Type II epithelial cells are critical target for hyperoxia-mediated impairment of postnatal lung development. Am J Physiol Lung Cell Mol Physiol 291: L1101–L1111, 2006. First published July 21, 2006; doi: 10.1152/ajplung.00126.2006.—Type II epithelial cells are essential for lung development and remodeling, as they are precursors for type I cells and can produce vascular mitogens. Although type II cell proliferation takes place after hyperoxia, it is unclear why alveolar remodeling occurs normally in adults whereas it is permanently disrupted in newborns. Using a line of transgenic mice whose type II cells could be identified by their expression of enhanced green fluorescent protein and endogenous expression of surfactant proteins, we investigated the age-dependent effects of hyperoxia on type II cell proliferation and alveolar repair. In adult mice, type II cell proliferation was low during room air and hyperoxia exposure but increased during recovery in room air and then declined to control levels by day 7. Eight weeks later, type II cell number and alveolar compliance were indistinguishable from those in room air controls. In newborn mice, type II cell proliferation markedly increased between birth and postnatal day 7 before declining by postnatal day 14. Exposure to hyperoxia between postnatal days 1 and 4 inhibited type II cell proliferation, which resumed during recovery and was aberrantly elevated on postnatal day 14. Eight weeks later, recovered mice had 70% fewer type II cells and 30% increased lung compliance compared with control animals. Recovered mice also had higher levels of T1α, a protein expressed by type I cells, with minimal changes detected in genes expressed by vascular cells. These data suggest that perinatal hyperoxia adversely affects alveolar development by disrupting the proper timing of type II cell proliferation and differentiation into type I cells.

alveoli; cell proliferation; differentiation; enhanced green fluorescent protein; proliferating cell nuclear antigen

THE ALVEOLUS IS COMPOSED OF TWO EPITHELIAL CELL TYPES THAT CAN BE IDENTIFIED BY THEIR DISTINCT MORPHOLOGY AND EXPRESSION OF UNIQUE GENES. TYPE I CELLS ARE THIN, FLAT CELLS THAT COVER PULMONARY VASCULAR ENDOTHELIAL CELLS AND COMPRIZE 95% OF THE ALVEOLAR SURFACE (56). THESE CELLS ARE IMPORTANT FOR GAS EXCHANGE, REGULATION OF ALVEOLAR FLUID LEVELS, AND STRETCH-INDUCED MODULATION OF SURFACANT SECRETION. TYPE II CELLS CAN BE IDENTIFIED BY THEIR EXPRESSION OF T1α (ALSO KNOWN AS RTIαo), AQUAPORIN-5, CAVESIN-1, OR THE CYCLIN-DEPENDENT KINASE INHIBITOR p15 (41, 42). TYPE II CELLS, ON THE OTHER HAND, ARE LARGE, CUBOIDAL CELLS THAT CONTAIN LAMELLAR BODIES AND APICAL MICROVILLI (21). THESE CELLS ARE IMPORTANT FOR PRODUCING SURFACANT, REGULATING ALVEOLAR FLUID LEVELS, AND HOST DEFENSE. TYPE II CELLS MAY BE IDENTIFIED BY THEIR EXPRESSION OF SURFACANT PROTEINS (SP)-A, SP-B, AND SP-C, OF WHICH SP-C IS EXPRESSED SOLELY BY TYPE II CELLS (54). TYPE II CELLS ARE ALSO IMPORTANT PROGENITOR CELLS FOR TYPE I CELLS DURING LUNG DEVELOPMENT AND REMODELING OF INJURED ALVEOLI.

Because in vivo exposure of adult animals to hyperoxia (oxygen >90%) selectively kills alveolar type I cells, it has often been used to study how type II cells participate in alveolar remodeling (3, 16, 26). Although type II cells swell during exposure, frank destruction is not observed by the time mortality occurs. During recovery in room air, type II cells proliferate, and some differentiate into type I cells (2, 51). A similar process occurs in vitro, where freshly isolated type II cells spontaneously lose expression of surfactant genes and gain expression of type I cell-specific markers (17, 48). Although SP expression can be restored by addition of keratinocyte growth factor and culture on Matrigel or floating collagen gels, it is unknown whether surfactant expression can be restored in vivo. Differentiating type II cells may also contribute to vascular development by their transient expression of vascular endothelial growth factor (VEGF) (30). Although alveolar structure is often restored, abnormal repair and fibrosis can occur when the proliferation of type II cells and fibroblasts becomes unbalanced (4). Fibrosis may be caused by the loss of type II cells, which express antimitogens for fibroblasts (40). Although cultured type II cells isolated from hyperoxic rats undergo apoptosis, extensive type II cell apoptosis after in vivo hyperoxia has yet to be reported (13). Nonetheless, restoration of normal alveolar structure requires proper proliferation and differentiation of type II cells.

Unlike adults that succumb to hyperoxia, newborns are remarkably tolerant and can survive for weeks in fairly high oxygen concentrations. However, hyperoxia disrupts postnatal alveolar development, and this may be a significant cause of bronchopulmonary dysplasia (BPD) seen in premature infants (10). As in infants with BPD, the lungs of newborn rats exposed to hyperoxia exhibit enlarged, simplified alveoli and overall growth arrest (9, 18, 52). Even brief exposure to hyperoxia followed by recovery in room air permanently disrupts alveolar development (33). Interestingly, low exposures of 0.4 inspired O₂ fraction for 6 days followed by recovery in

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room air were sufficient to permanently retard lung development in newborn rats (12). Despite great efforts by many investigators, it remains unclear how hyperoxia disrupts alveolar development. Because postnatal lung development requires coordinated proliferation and differentiation between epithelial and mesenchymal cells, hyperoxia is likely to disrupt paracrine signaling. Indeed, exogenous VEGF restores alveolar development in newborn rats exposed to hyperoxia for 12 days and recovered in room air for an additional 10 days (28).

Consistent with VEGF being a critical regulator of lung development, pharmacological inhibitors of VEGF receptors disrupt alveolar development in mice or rats (29, 34). In addition to disrupting vascular signaling, hyperoxia also inhibits cell proliferation, increases cell death, and promotes inflammation, all of which could contribute to disrupted lung development (6, 20, 35, 44). Despite evidence that hyperoxia disrupts type II cell proliferation and organization in premature baboons (31, 50), little is known about how these cells participate in the abnormal remodeling observed when newborns are recovered in room air.

One of the limitations of studying cell fate during tissue injury is that cell-specific gene expression frequently declines. For example, hyperoxia inhibits expression of pro-SP-B and pro-SP-C that would otherwise be used to identify type II cells in tissue sections (47, 55). For these reasons, we created a line of transgenic mice in which enhanced green fluorescence protein (EGFP) was targeted to type II cells under control of the human SP-C gene promoter (47). We reasoned that the intrinsic green fluorescence of EGFP would be retained longer during hyperoxia because it is not secreted. As hypothesized, type II cells could be identified by their expression of EGFP in adult mice exposed to hyperoxia even when SP-C immunostaining had declined (47). In the present study, these mice were used to investigate type II cell proliferation and alveolar remodeling in adult and newborn mice exposed to hyperoxia. Unlike adult lungs that fully recover from hyperoxia, newborn lungs recovered from hyperoxia display a significant reduction in the number of type II cells along with increased alveolar compliance. Our findings provide new insight into how neonatal hyperoxia permanently disrupts alveolar development.

MATERIALS AND METHODS

Mouse and oxygen exposures. The generation and characterization of transgenic mice expressing EGFP in type II cells under control of the human SP-C gene promoter have been described (47). The transgene is maintained on the C57BL/6j background as a homozygous line of mice. Adult (8–12 wk old) transgenic mice were exposed to room air or hyperoxia (100% oxygen) for 64 h and recovered in room air for 8 wk (38). Two hours before death, mice were injected with 1% (vol/wt) 5-bromo-2′-deoxyuridine (BrdU) labeling reagent (Zymed Laboratories, San Francisco, CA). Newborn mice were born into room air in order for maternal interactions with the pups to form. On postnatal day 1, pups were randomly separated into two groups. One group remained in room air, and the other was exposed to hyperoxia for 4 days. Mothers were rotated between the two groups every 24 h. After 4 days of hyperoxia, pups were returned to room air and rotation of the mothers between litters was ended. Animals were euthanized by intraperitoneal injection with 100 mg/kg pentobarbital sodium. The University of Rochester’s University Committee on Animal Resources (UCAR) reviewed and approved these studies.

Immunohistochemistry. Lungs were inflation fixed with 10% neutral buffered formalin. Lobes were dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin. Paraffin sections (4 μm) were deparaffinized with xylene and rehydrated through graded ethanol and water. BrdU was detected in lung sections with a BrdU staining kit (Zymed Laboratories) with 3,3′-diaminobenzidine as a substrate and methyl green as a counterstain. Sections for proliferating cell nuclear antigen (PCNA) immunostaining were subjected to antigen retrieval by boiling in 50 mM Tris pH 9.5 and incubated overnight in biotinylated anti-PCNA antibody (Zymed), and immune complexes were detected with streptavidin-Texas Red (Jackson Immunoresearch, West Grove, PA). EGFP was detected by visualizing intrinsic green fluorescence or with a fluorophore-conjugated goat-anti-EGFP antibody (1:1,000; Novus Biologicals, Littleton, CO). Because antigen retrieval inactivates EGFP fluorescence, pro-SP-B and pro-SP-C were detected with rabbit anti-pro-SP-B (1:1,000) or -pro-SP-C sera (1:10,000; Chemicon International, Temecula CA) and a Tyramide Signal Amplification biotin system. Clara cell secretory protein (CCSP) was detected with rabbit anti-CCSP sera (43, 47). T1 was detected with hamster anti-mouse monoclonal T1c (1:1,000; clone 8.1.1, Iowa Hybridoma Bank). After tissues were immunostained, sections were immersed in 4′,6-diamidino-2-phenylindole (DAPI) and visualized with a Nikon E-800 fluorescence microscope (Nikon, Melville, NY). Images were captured with a SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Quantitative immunohistochemistry. Images of random, noncontiguous fields of parenchyma were acquired with a Nikon E-800 microscope equipped with a SPOT-RT camera. Five fields per lung were obtained from at least three separate animals for each treatment. Fields that contained a large airway or blood vessel were rejected. For counting BrdU-positive cells, regions were selected under bright-field illumination. For counting fluorescence-positive cells, regions were selected under DAPI fluorescence to prevent bias toward fields with FITC or Texas Red signals. Different fluorescent filters were used to acquire images of each field displaying nuclei (DAPI), PCNA or pro-SP-C (Texas Red), and pro-SP-B or EGFP (FITC). Images were digitally merged to identify dual-positive cells. Quantification was performed with Metamorph software (Universal Imaging, Downingtown, PA). Metamorph was configured to measure total nuclei based on the average number of pixels in a nucleus. For each animal, the counts from all fields were summed and the following ratios were determined: BrdU positive/nuclei, pro-SP-B positive/DAPI positive, EGFP positive/DAPI positive, pro-SP-C positive/DAPI positive, PCNA positive/DAPI positive, PCNA positive/pro-SP-B positive, and EGFP positive/EGFP positive cells. The ratios for all animals at each time point were averaged and graphed.

Western blots. Tissues were homogenized in lysis buffer containing protease inhibitors (38). Proteins were separated on polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Membranes were blocked in 5% nonfat dry milk before incubating overnight at 4°C in anti-EGFP (1:1,000; Clontech, Palo Alto, CA), anti-pro-SP-C (1:1,000; Santa Cruz Biotechnology), anti-platelet endothelial cell adhesion molecule (PECAM) (1:1,000; Santa Cruz Biotechnology), anti-T1a (1:2,000; Iowa Hybridoma Bank), or β-actin (1:1,000; Sigma, St. Louis, MO) sera. Immune complexes were detected by chemiluminescence (Amersham, Arlington Heights, IL) and visualized by exposure to Kodak Bio-Max film. Images were captured on a FluorChem SP (Alpha Innotech, San Leandro, CA), and band intensities were quantified.

RNase protection assays. Total RNA was prepared from lung homogenates with TRIzol (Invitrogen). Radiolabeled RNA probes encoding Flt1, Flt4, Tie1, Tie2, CD31, VEGF, endoglin, and L32 were synthesized at room temperature according to the RiboQuant Multi-Probe RNase Protection Assay System (PharMingen, San Diego, CA), hybridized to 10 μg of total RNA, and processed according to the kit (37). Protected RNAs obtained after RNase digestion were ethanol precipitated and separated on a 6% polyacrylamide gel. The gel was dried and exposed to a Phospholmage screen for visualization. Band intensities were normalized to L32 and quantified with ImageQuant analysis.
Statistical analysis. Values are expressed as means ± SD unless noted. Group means were compared by ANOVA with Fisher’s procedure for post hoc analysis, and $P < 0.05$ was considered significant.

RESULTS

Type II cell proliferation in adult mice recovered from hyperoxia. Adult 8-wk-old mice were exposed to room air or hyperoxia for 64 h and then recovered in room air for 10 days. This exposure-recovery model allows for significant alveolar injury and repair with minimal animal mortality. Mice were injected with BrdU 2 h before death, and cell proliferation was assessed by counting BrdU-positive cells in lung sections. BrdU-positive alveolar cells were rarely detected in mice exposed to room air and declined further during hyperoxia, although this was not statistically significant (Fig. 1A). The proportion of BrdU-positive cells increased sevenfold after 72 and 120 h of recovery before returning to control levels by 168 h. These changes in cell proliferation were confirmed by staining sections for PCNA. PCNA is an auxiliary protein of DNA polymerases delta and epsilon that encircles the DNA helix and is essential for DNA replication. PCNA-positive cells were rarely detected in mice exposed to room air and declined further during hyperoxia, although this was not statistically significant (Fig. 1B). The proportion of PCNA-positive cells markedly increased seven- to eightfold in mice recovered from hyperoxia before declining to control levels by 168 h. Thus cell proliferation is very low in adult mice while markedly and transiently increasing during recovery in room air.

We created a line of transgenic mice that express EGFP in a subpopulation of type II cells (47). By colocalizing EGFP with pro-SP-B, we were able to determine the proportion of type II cells that express EGFP. Airway cells, which also express pro-SP-B, were excluded from this analysis. EGFP was detected in 1.8 ± 0.4% (means ± SE) of all alveolar cells. Pro-SP-B was detected in 20.7 ± 1.0% of all alveolar cells, of which 7.4 ± 1.9% exhibited green fluorescence. Thus EGFP defines a subset of type II cells. To identify proliferating type II cells, PCNA was colocalized to EGFP. In adult mice exposed to room air, PCNA was detected in 1% of EGFP-positive type II cells (Fig. 2). Type II cell proliferation remained low on the first day of recovery, markedly increased between 72 and 120 h, and then declined. Consistent with increased type II cell proliferation during recovery, PCNA was also detected in 30–40% of pro-SP-B-positive alveolar cells of adult mice recovered from hyperoxia for 3 days (data not shown). These data confirm that type II cell proliferation increases during recovery from hyperoxia (2, 25).

The long-term effects of type II cell proliferation on alveolar remodeling were assessed in adult mice that were exposed to hyperoxia for 64 h and then recovered in room air for 8 wk. The average number of DAPI-positive cells counted per field in mice exposed to room air (260.7 ± 57.8) was not significantly different from that in mice recovered from hyperoxia (251.7 ± 59.4) ($P = 0.65$). Likewise, the proportion of type II cells after 8 wk of recovery (1.8 ± 0.9) was not significantly different...
from that in mice recovered from hyperoxia (1.9 ± 0.9) (P = 0.96). Western blot analysis of lung homogenates confirmed that expression of EGFP was not different (data not shown). These findings support the concept that type II cell proliferation and differentiation contribute to normal remodeling of adult mice recovering from hyperoxia.

Type II cells are reduced in newborn mice recovered from hyperoxia. Because alveolar development is permanently disrupted in newborn mice recovered from hyperoxia, the effects of hyperoxia on type II cell proliferation were investigated. Newborn mice were exposed to hyperoxia from postnatal day 1 to postnatal day 4 and then recovered in room air for up to 8 wk. To ensure that outcomes were not attributed to differences in maternal care, mothers were rotated between pups exposed to room air and hyperoxia. Despite this, the average weight of newborn mice recovered from hyperoxia for 1 and 2 wk was 50% and 25% less, respectively, than that of their siblings exposed to room air (n = 70; P < 0.001). By 4 wk of recovery, animal weights were not significantly different. At 8 wk of age, the average weight of mice exposed to room air was 21.8 ± 2.1 g vs. 20.2 ± 2.6 g for mice exposed to and recovered from hyperoxia (n = 18; P = 0.23). These data are consistent with studies showing that newborn exposure to hyperoxia inhibits cell proliferation and overall animal growth, which returns to control levels during recovery in room air (33).

Surprisingly, adult lungs previously exposed to hyperoxia as newborns contained few EGFP-positive type II cells (Fig. 3A). Moreover, fluorescence intensity was less in positive cells. To identify when hyperoxia reduced the number of type II cells, the proportion of EGFP-positive cells was quantified. The proportion of EGFP-positive cells was very low at birth and progressively increased with age, reaching a maximum of 6% by 6 wk before slightly declining at 8 wk. In newborns exposed to hyperoxia, the proportion of EGFP-positive cells was not different until the second week of recovery, at which time it failed to increase at the same rate as room air controls. Because intrinsic green fluorescence intensity was also less in mice recovered from hyperoxia, there was concern that faint green type II cells were not being counted. To amplify the EGFP signal such that all positive cells exhibited the same fluorescence intensity, sections were stained with an anti-EGFP antibody that was detected with Texas Red-conjugated secondary antibodies. The total numbers of intrinsic green EGFP- and red immunostained EGFP-positive cells were quantified and found to be not significantly different.

To confirm that the effects of hyperoxia on EGFP-expressing type II cells reflected all type II cells, the proportion of alveolar cells expressing pro-SP-B was determined. Airway epithelial cells, which also express pro-SP-B, were excluded from this analysis. The proportion of pro-SP-B-positive cells was ~4% at birth and progressively increased with age until 25–30% of alveolar cells were pro-SP-B positive in adults (Fig. 3B). In newborn mice recovering from hyperoxia, the number of pro-SP-B-positive cells increased at a slower rate beginning at 2 wk of age and remained low through adulthood.

The expression of pro-SP-C was also investigated because its expression is restricted to type II cells. Because of high background staining in newborn mice, we were only able to quantify pro-SP-C-positive cells in adult lungs. Like EGFP and pro-SP-B, the number and staining intensity of pro-SP-C-positive cells were significantly less in adult mice recovered from newborn hyperoxia (Fig. 4A). Pro-SP-C was detected in 20.7 ± 5.1 cells of 8-wk-old mice exposed to room air vs. 14.2 ± 3.3 cells of mice recovered from newborn hyperoxia (P < 0.001). Despite fewer type II cells in recovered mice, the total number of DAPI-positive cells counted was not significantly different (P = 0.34). This suggested that hyperoxia may have stimulated the differentiation of type II cells into type I cells rather than simply killing off type II cells. Consistent with this hypothesis, alveolar staining of T1α, a gene expressed by type I cells, was markedly elevated in adult mice that had been exposed to hyperoxia as newborns (Fig. 4B). Moreover, <1% of alveolar cells exhibited terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining.
During postnatal lung development, and this low level of apoptosis was not increased after exposure to hyperoxia (data not shown).

Western blot analysis was used to confirm differences seen in antibody staining (Fig. 5A). Recovered mice expressed less EGFP or pro-SP-C and more T1α. Quantitative analysis of band intensities confirmed that recovered mice expressed fold changes of 2.86 ± 0.55 ($P < 0.007$) of T1α, 0.65 ± 0.09 ($P < 0.008$) of EGFP, and 0.23 ± 0.04 ($P < 0.00005$) of pro-SP-C compared with unexposed controls. Because type II cells are a significant source of VEGF, an important mitogen for vascular development, the expression of vascular-specific proteins was also investigated. Surprisingly, the expression of PECAM was not different ($P < 0.38$). Likewise, mRNA expression of the vascular genes Flt1, Flt4, Tie1, Tie2, thrombin receptor (TR), PECAM, endoglin, or VEGF was not significantly different between the mice (Fig. 5B). VEGF protein was detected in airway epithelial and alveolar type II cells by immunostaining (data not shown). Because staining intensity was comparable between adult mice exposed to room air versus recovered from hyperoxia, it is unlikely that fewer type II cells compensated by expressing more VEGF.

**Hyperoxia disrupts type II cell proliferation in newborn mice.** Because fewer type II cells were detected in adult mice recovered from newborn hyperoxia, cell proliferation was investigated by counting PCNA-positive cells. The proportion of PCNA-positive alveolar cells was low at birth, markedly increased on postnatal day 4, and then declined over time until a steady-state level of ~1% was reached at 4 wk of age (Fig. 6A). Cell proliferation was significantly inhibited in postnatal day 4 mice exposed to hyperoxia. However, it markedly increased at 1 wk of recovery and then declined until it reached

![Fig. 3.](http://www.ajplung.org/)

Newborn exposure to hyperoxia disrupts the proportion of alveolar type II cells detected during postnatal lung development. Newborn mice were exposed to room air or hyperoxia for 4 days and recovered in room air. Sections were immunostained for Clara cell secretory protein (red) with intrinsic EGFP (green) fluorescence (A) or for pro-surfactant protein (SP)-B (red) (B). Representative images are taken from 8-wk-old adult mice that had been exposed continuously to room air or recovered from hyperoxia. The proportion of positive cells was determined and graphed. Values represent means ± SD. *$P < 0.05$ relative to room air controls of the same age. PND, postnatal day.
a steady state of ~0.5% at 6 wk of age. This low level of cell proliferation was significantly less than that detected in mice exposed to room air.

Proliferating type II cells were identified in cells expressing both PCNA and EGFP. Unfortunately, it was not possible to identify dual-positive cells because EGFP was not highly expressed in newborn mice. Moreover, EGFP-positive cells were significantly reduced in newborn mice recovered from hyperoxia. However, proliferating type II cells were identified based on their expression of both PCNA and pro-SP-B (Fig. 6B). Type II cell proliferation was low at birth and markedly increased over the first week of life. It then significantly declined and reached a steady-state level between the first and second weeks. In contrast, type II cell proliferation was inhib-
Hyperoxia inhibits type II cell proliferation and functions of newborn mice. On postnatal day 7, type II cell proliferation was significantly higher in room air than in hyperoxia. However, by postnatal day 14, type II cell proliferation was significantly lower in room air than in hyperoxia, indicating that type II cell proliferation is inhibited by hyperoxia and that it resumes during the recovery period. These data show that hyperoxia temporarily shifts type II cell proliferation from postnatal day 7 to day 14.

Hyperoxia permanently disrupts alveolar function in newborn mice. Because adult mice that were exposed to hyperoxia as newborns had fewer type II cells, pulmonary function tests were performed to see whether these changes affected lung physiology (Fig. 7A). Although airway resistance was lower in recovered mice, the difference did not reach statistical significance (P = 0.16). In contrast, alveolar compliance was significantly higher by 30% (P < 0.004). Increased compliance is indicative of larger alveoli, which was significantly elevated in adults that had been exposed to hyperoxia as newborns (33). To confirm that these changes were attributed to newborn exposure to hyperoxia, airway resistance and alveolar compliance were measured in adult mice that had been exposed to and recovered from hyperoxia as adults (Fig. 7B). Unlike newborn exposure, adult exposure to hyperoxia did not alter airway resistance or alveolar compliance (P < 0.83 for both analyses).

**DISCUSSION**

This study compared type II cell proliferation and alveolar remodeling in adult and newborn mice recovering from hyperoxia. Although type II cell proliferation was not readily detected in adult mice, it transiently increased during the recovery period and contributed to normal alveolar remodeling. In contrast, type II cell proliferation in newborn mice was low at birth, increased during the first week, and then declined by postnatal day 14 to the low levels seen in adults. Despite being inhibited by hyperoxia, type II cell proliferation resumed during the recovery period and was aberrantly increased on
A number of studies suggest that alveolar development is closely coordinated with growth of the pulmonary circulation. For example, exposure of adult rabbits or mice to hyperoxia reduces expression of VEGF (8, 53). Likewise, expression of VEGF and VEGF receptor Flt-1 declined in premature baboons and human infants that developed chronic lung disease attributed to high oxygen exposure (8, 32). Consistent with the concept that loss of VEGF contributes to the inhibition of alveolar development, normal alveolar development could be restored in hyperoxic newborn rats by providing VEGF exogenously (28). Conversely, treatment of newborn rats with the VEGF receptor inhibitors SU-5416 or DC-101 decreased alveolarization and vascular growth (29, 34). These data collectively support the “vascular hypothesis” concept in which impaired VEGF signaling contributes to the pathogenesis of lung disease such as BPD (1). Because type II cells are a significant source of VEGF, their loss as shown in the present study helps explain how VEGF levels decline during hyperoxia and why exogenous VEGF is efficacious. The mechanisms by which VEGF protects the developing lung against hyperoxia remain to be clarified. VEGF is clearly an important prosurvival and vascular-specific mitogen for endothelial cells, which express VEGF receptors Flt-1 and Flk-1. Because endothelial cells are highly sensitive to oxidant stress, the loss of VEGF may contribute to their susceptibility to hyperoxia. However, type II cells may also express Flk-1, and VEGF has been shown to stimulate SP-B and SP-C expression (15). Although a direct role of VEGF signaling on type II cells remains to be confirmed, attenuating VEGF signaling in lung renal capsule grafts significantly affects lung epithelial cell development (57). Thus VEGF is clearly important for both endothelial and epithelial cell growth and survival.

Given the importance of vascular cells for lung development, it was surprising not to detect permanent changes in vascular-specific genes in adult mice recovered from newborn hyperoxia. It does not appear from immunohistochemical staining that type II cells in recovered adult mice compensated by expressing more VEGF (data not shown). As shown in the Western blot in Fig. 5 and as defined by immunohistochemical staining, PECAM (CD31) levels were also not different (data not shown). Although additional studies using transmission electron microscopy are needed to quantify endothelial cells, these data collectively suggest that the number of endothelial cells may be similar in recovered mice. One trivial explanation for why vascular genes appear to be less affected than reported in animal models of BPD is that our newborn mice were exposed to 4 days of hyperoxia, which is relatively short compared with other newborn animal models. Indeed, disrupted vascular development was observed in infants that had died, in part, from their exposure to prolonged hyperoxia. Consistent with this hypothesis, expression of VEGF mRNA was unaltered in newborn mice exposed to hyperoxia for 4 days (data not shown). Additionally, mice were recovered in room air for 8 wk, which is relatively long compared with other recovery models. Considering the importance of the vasculature for lung development, it will be important to investigate further the role of VEGF and other vascular-specific signaling pathways during this recovery period.

The loss of type II cells in newborn mice recovered from hyperoxia may help clarify why infants who survive BPD often...
return as children with respiratory problems. BPD is the most common form of chronic lung disease in the newborn and is frequently seen in premature infants with very low birth weight (22). The lungs of these infants are less vascularized, and alveoli are fewer and larger. Because premature infants have low plasma levels of glutathione and develop BPD when exposed to hyperoxia and ventilation, it is generally accepted that BPD is caused largely by oxidative stress to the developing lung (49). If we can extrapolate our findings with BPD, the inability of type II cells to proliferate during the first week of life may permanently alter postnatal lung growth during a critical period of postnatal lung development. For those who survive BPD, the long-term detrimental effects of oxidative stress in the neonatal period are just beginning to be appreciated. Sadly, survivors exhibit increased airway resistance and reduced lung capacity later in life (45). These changes have even been reported in children as old as 8–10 yr of age (5). Clinical studies reveal that exposure to oxygen in the neonatal period is associated with permanent narrowing of small airways (14). Perhaps one of the most compelling arguments that oxidative stress can alter human lung development comes from clinical studies in which premature infants instilled with recombiant CuZn superoxide dismutase had improved pulmonary function at 1 yr of corrected age (19). In the present study, newborn exposure to hyperoxia did not significantly alter airway resistance or morphology in recovered adult mice. A trivial explanation is that airways of premature infants are at a different stage of development than those of normal newborn mice. In other words, premature airways may be more sensitive to oxidative stress than term airways. Alternatively, recovered mice might have outgrown an airway deficit that would have been seen at an earlier age. Nonetheless, our finding that recovered mice have fewer type II cells suggests that similar losses may be taking place in conducting airways, particularly in premature humans who are already sensitive to oxidative stress.

There are several limitations to this study. First, adult mice were exposed to hyperoxia for 64 h, whereas newborns were exposed for 4 days. The differences seen in lung remodeling are unlikely to be caused by the length of exposure or the degree of lung injury. Even though adults recover, they are more injured after 64 h of hyperoxia than newborns exposed for 4 days. Second, type II cells were quantified based on their expression of EGFP, pro-SP-B, or pro-SP-C. Although less staining was interpreted as fewer cells, it remains possible that individual type II cells simply express less protein that could not be detected and thus the total number of type II cells is actually unchanged. At this time, our data cannot dispute the possibility that the total number of type II cells in the entire lung is unchanged. The use of three independent markers for type II cells, the compensatory increase in expression of the type I cell specific protein T1α, and the fact that the total number of cells counted was not different between the mice makes this seem unlikely. Clearly, morphometric counts of type I cells by transmission electron microscopy are needed to confirm that recovered lungs have more type I cells. Although we did not detect increased TUNEL staining, we cannot rule out that apoptosis may also contribute to the reduced numbers of type II cells seen in recovered mice. Indeed, increased TUNEL staining has been seen in newborn mice exposed to hyperoxia, and freshly isolated embryonic mouse type II cells apoptosis when exposed to hyperoxia (7, 20, 35). Because TUNEL staining may reflect oxidative DNA strand breaks and not necessarily apoptosis (7, 46), additional studies are needed to clarify the fate of type II cells in these recovered mice. Finally, the thin morphology of microvascular endothelial and type I epithelial cells makes them difficult to quantify. Although differences in T1α expression were readily detected, it remains possible that recovered lungs have subtle changes in microvascular cells that cannot be detected by analyzing the entire lung.

In summary, short-term exposure of newborn mice to hyperoxia permanently disrupts alveolar development as defined by a proportional loss in the number of type II cells and increased alveolar compliance possibly caused by compensatory increase in the number of type I cells. These effects are attributed to a temporal shift in type II cell proliferation from postnatal day 7 to day 14. In light of these findings, we speculate that this shift causes type II cells to be proliferating in an environment that now favors or accelerates their differentiation to type I cells. This disruption in alveolar epithelial cell differentiation might contribute to the pathological processes seen in infants with BPD and perhaps adults who develop emphysema and chronic obstructive pulmonary disease.

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