Neonatal Hyperoxia Increases Sensitivity of Adult Mice to Bleomycin-Induced Lung Fibrosis

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Supplemental oxygen used to treat infants born prematurely constitutes a major risk factor for long-term deficits in lung function and host defense against respiratory infections. Likewise, neonatal oxygen exposure results in alveolar simplification in adult mice, and enhances leukocyte recruitment and fibrosis when adult mice are infected with a sublethal dose of influenza A virus. Because pulmonary fibrosis was not observed in infected adult mice exposed to room air as neonates, previous neonatal oxygen exposure may have reprogrammed how the adult lung responds to epithelial injury. By administering bleomycin to adult mice exposed to room air or hyperoxia as neonates, we tested the hypothesis that neonatal hyperoxia enhances fibrosis when the epithelium is injured by direct fibrotic stimulus. Increased sensitivity to bleomycin-induced lung fibrosis was observed in adult mice exposed to neonatal hyperoxia, and was associated with increased numbers of leukocytes and an accumulation of active transforming growth factor (TGF)- β 1 in the lung. Fate mapping of the respiratory epithelium revealed that the epithelial-mesenchymal transition was not a significant source of fibroblasts in room air-exposed or oxygen-exposed mice treated with bleomycin. Instead, the treatment of mice with anti-Gr-1 antibody that depletes neutrophils and myeloid-derived suppressor cells reduced the early activation of TGF- β 1 and attenuated hyperoxia-enhanced fibrosis. Because bleomycin and influenza A virus both cause epithelial injury, understanding how neonatal hyperoxia reprograms the epithelial response to these two different injurious agents could lead to new therapeutic opportunities for treating lung diseases attributed to prematurity.

Keywords: epithelial injury; neonatal; neutrophils; pulmonary fibrosis; Type II cells

Premature infants are at risk for developing bronchopulmonary dysplasia (BPD), a chronic form of lung disease attributed to an arrest in postnatal lung development (1). Fortunately, the use of antenatal steroids, milder ventilation strategies, and exogenous surfactant has improved the survival of these infants. However, the prevalence of pulmonary and other organ complications attributed to prematurity has not changed (2, 3). Children born prematurely often display reduced pulmonary function and lung capacity, and are at increased risk for respiratory viral infections and asthma (4–7). They also demonstrate a higher incidence of retinopathy, neurodevelopmental delay, and social behavior

CLINICAL RELEVANCE

Supplemental oxygen administered to infants born prematurely constitutes a major risk factor for chronic lung disease later in life. We reveal that neonatal hyperoxia enhances leukocyte recruitment into the airspace, the activation of transforming growth factor- β 1, and lung fibrosis in adult mice receiving bleomycin. Understanding how neonatal hyperoxia reprograms the cellular response to epithelial injury could lead to new opportunities for enhancing the health of individuals born prematurely.

issues (8, 9). Such changes have been attributed to early-life exposure to oxygen and cytotoxic reactive oxygen species that injure or reprogram the development of the lungs, eyes, and brain. The lungs of preterm infants are especially at risk for oxidative damage, because antioxidant defenses do not mature until late in gestation (10, 11). Consistent with this concept, oxygen supplementation in the neonatal period has been directly associated with altered lung function later in life (12, 13). Moreover, the treatment of preterm infants with recombinant human superoxide dismutase improved pulmonary function and reduced hospitalization rates at 1 year of corrected age (14). Hence, an urgent need exists to understand how prematurity and early-life exposure to oxygen affect health later in life.

High oxygen exposure in preterm baboons and sheep and newborn rodents promotes alveolar simplification and lung dysfunction, much like those reported in preterm infants who died from complications of BPD (15–17). Such changes may be permanent because alveolar simplification persists even during recovery in room air (18, 19). To understand how oxygen permanently reprograms lung development, we developed a model wherein newborn mice were exposed to room air or 100% oxygen (hyperoxia) between postnatal Days 0 and 4, and then the oxygen-treated mice were returned to room air. We discovered that neonatal hyperoxia causes lung simplification by 8 weeks of age, which was associated with a reduction in the number of pro-surfactant protein C (pro-SP-C)-positive alveolar epithelial Type II cells and an increase in genes expressed by alveolar epithelial Type I cells (20). Moreover, infection with a sublethal dose of influenza A virus resulted in heightened inflammatory responses in young adult mice exposed to hyperoxia as neonates, compared with their infected siblings that had been exposed to room air as neonates (21, 22). Pulmonary fibrosis, as defined by α -smooth muscle actin-positive myofibroblasts and the collagen staining of honey-combed structures, was evident by Day 14 after infection in mice exposed to hyperoxia as neonates, but not in their siblings that had been exposed to room air. Because pulmonary fibrosis is rarely seen in people infected with influenza A virus, neonatal hyperoxia may have reprogrammed how the lung implements repairs when the epithelium is injured.

To test whether neonatal hyperoxia reprograms how the lung responds to an epithelial injury that results in fibrosis, we administered bleomycin to adult mice that had been exposed to room air or hyperoxia as neonates. Bleomycin is an antineoplastic drug

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that causes DNA double-strand breaks in alveolar epithelial Type I cells and microvascular endothelial cells (23, 24). It also impairs Type II cell proliferation, and hence the normal repair of an injured alveolus (25). Here we report how exposure to neonatal hyperoxia enhances the fibrotic effects of bleomycin by rapidly enhancing the recruitment of leukocytes (particularly neutrophils) to the lung, a phenomenon that was not observed when hyperoxia-exposed mice were infected with influenza A virus (21). Understanding how early-life exposure to hyperoxia reprograms how the lung responds to bleomycin is important, because it may clarify how individuals born prematurely respond to different types of respiratory insults. Some of these findings were presented at the American Thoracic Society meeting in San Francisco (May 2012).

MATERIALS AND METHODS

More detailed materials and methods may be found in the online supplement.

Exposure of Mice to Hyperoxia and Bleomycin

Newborn mice were exposed to room air or 100% oxygen between postnatal Days 0–4, and recovered in room air until they were 8 weeks old (20). A single dose of bleomycin (2.5 U/kg) in 50 μ l of PBS was administered to young adult (8–12 wk old) male mice by tracheal instillation. Some mice were injected intraperitoneally with 300 μ g of anti-Gr-1 antibody or with 300 μ g of rat IgG as control (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The University Committee on Animal Resources at the University of Rochester approved the use of mice for these studies.

Collagen Assay

Total lung collagen was determined using the Sircol Collagen Assay kit (Biocolor, Ltd., Belfast, Northern Ireland, UK) according to the manufacturer's instructions and as previously described (22).

Immunohistochemistry

Lungs were inflation-fixed with 10% neutral-buffered formalin (20, 27). Sections were stained with antibodies against enhanced green fluorescence protein (EGFP) or anti- α -smooth muscle actin, as described elsewhere (20, 21), or phospho-Smad3 (1:50; Cell Signaling, Danvers, MA), or S100A4 (1:100; Thermo Scientific, Rockford, IL). Collagen was detected using Gomori's trichrome stain (Richard-Allan Scientific Co., Kalamazoo, MI).

Quantification of Leukocytes in Bronchoalveolar Lavage Fluid

The total number of leukocytes and percentages of macrophages, lymphocytes, and neutrophils were determined by counting cells obtained in bronchoalveolar lavage (BAL) fluid (22).

Myeloperoxidase Activity Assay

Right lung lobes were sonicated in fresh chilled 0.5% hexadecyltrimethylammonium bromide in potassium phosphate buffer (pH 6) per milligram of tissue. Samples were centrifuged at $13,000 \times g$, and myeloperoxidase (MPO) activity in the supernatant was determined at 460 nm, using commercially available MPO (Sigma Chemical Co., St. Louis, MO) to generate a standard curve (26).

RNA Isolation and Quantitative RT-PCR

Total RNA isolated from the right lung lobe was reverse-transcribed, using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). cDNA was then amplified with SYBR Green I dye on the CFX96 Touch and CFX384 Touch Real-Time PCR detection system (Bio-Rad Laboratories). PCR products were amplified with murine TGF- β 1

(forward primer, 5'-TACGTCAGACATTCGGGAAGCA-3'; reverse primer, 5'-AGGTAACGCCAGGAATTGTTGC-3') and murine 18s ribosomal RNA (forward primer, 5'-CGGCTACCACATCCAAGGAA-3'; reverse primer, 5'-GCTGGAATTACCGCGGCT-3') primers, which were used to normalize equal loading of the template cDNA.

Quantification of TGF- β 1 Protein

Concentrations of active and total (latent plus active) TGF- β 1 protein in BAL fluid were determined using the murine TGF- β 1 DuoSet ELISA Development System (R&D Systems, Minneapolis, MN).

Statistical Analyses

All studies were performed at least three times with at least three mice per outcome. Values are expressed as means $\pm$ the standard error of a single experiment. Group means were compared by ANOVA, followed by a Fisher least significant difference *post hoc* test. Body weight differences of the mice before exposure to hyperoxia were analyzed by an unpaired Student *t* test. Survival was evaluated using the Kaplan-Meier test and analyzed for significance by the Mantel-Cox test. All data were analyzed using StatView software (SAS Institute, Cary, NC), and the means were considered significantly different when $P < 0.05$.

RESULTS

Some of the results are further detailed in the online supplement.

Neonatal Hyperoxia Increases Sensitivity of Adult Mice to Bleomycin-Induced Lung Fibrosis

Bleomycin (2.5 U/kg) was administered intratracheally to adult male mice that had been exposed to room air or 100% oxygen (hyperoxia) between Postnatal Days 0–4. This dose of bleomycin was chosen based upon pilot studies demonstrating minimal mortality in room air-exposed mice (data not shown). Before treatment with bleomycin, the mean body weight of adult mice exposed to hyperoxia was significantly less than that of siblings exposed to room air ($n = 10$ per group, $P < 0.0001$ according to unpaired Student *t* test) (Figure 1A). After treatment with bleomycin, the mean body weight of the room air-exposed mice declined over the first 12 days, and then increased to near pretreatment concentrations by Day 28, which was not significantly different from the starting weights ($P = 0.15$). Although the mean percent body weight of hyperoxia-exposed mice also declined and then increased, it was still significantly less than pretreatment weights by Days 26 and 28 ($P < 0.02$). This was readily apparent when weight change was graphed as percent weight loss (Figure 1B). When the mean percent change in body weight was analyzed between the two exposure groups over the entire course of 28 days, neonatal hyperoxia significantly reduced the percent body weight in mice treated with bleomycin compared with bleomycin-treated room air mice ($P < 0.0007$, according to an unpaired Student *t* test). Neonatal hyperoxia also significantly increased the mortality of mice treated with bleomycin through Day 25 ($P < 0.03$) (Figure 1C). The death of one room air-exposed mouse on Day 26 reduced the significance in survival to $P = 0.10$.

Total soluble collagen was quantified in a separate group of mice. Collagen abundance remained constant in room air-exposed mice treated with bleomycin through Day 21, and then increased significantly on Day 28 (Figure 2A). Concentrations of collagen in hyperoxia-exposed mice were similar to those found in room air-exposed mice on post-treatment Days 0, 7, and 14, and then increased significantly on Days 21 and 28, and these increases were greater than those observed in room air-exposed mice. The trichrome staining of lung tissue harvested from the same group of mice confirmed that exposure to neonatal hyperoxia increased lung collagen abundance and fibrosis

Figure 1. Neonatal hyperoxia increases bleomycin-induced morbidity and mortality. Bleomycin was administered to adult mice exposed to room air (RA) or hyperoxia (O₂) as neonates ($n = 10$ mice per group). (A) Mice were weighed before treatment with bleomycin (Day 0) and every other day after treatment for the next 28 days. (B) The mean percent body weight lost in mice exposed to hyperoxia was significantly greater on Days 26 and 28 ($*P < 0.02$), compared with siblings exposed to room air. (C) Survival of adult mice exposed to room air or hyperoxia as neonates, after treatment with bleomycin ($P < 0.10$).

(Figure 2B). Congestion and lung disease were grossly evident in all lung lobes of hyperoxia-exposed mice treated with bleomycin (Figure 2C). Lung disease was less evident in room air-exposed mice treated with bleomycin.

The Epithelial–Mesenchymal Transition Is Not a Significant Contributor to Bleomycin-Induced Lung Fibrosis

To examine the potential contributions of the epithelial–mesenchymal transition (EMT) in the altered response to neonatal hyperoxia, the respiratory epithelium was durably labeled with EGFP by mating Sftpc-Cre with mT/mG transgenic mice (see the online supplement for details). Newborn mice were exposed to room air or hyperoxia, and then treated with bleomycin as adults. Lungs were harvested 21 days later and stained for EGFP and

α -smooth muscle actin (α -SMA), a protein expressed by activated myofibroblasts, or S100A4, a protein expressed by fibroblasts. Epithelial cells expressing EGFP did not express α -SMA or S100A4, suggesting that the EMT is not a significant cause of bleomycin-induced lung fibrosis in adult mice exposed to room air or hyperoxia as neonates (Figure E1 in the online supplement).

Neonatal Hyperoxia Enhances the Recruitment of Leukocytes to Lungs of Adult Mice Treated with Bleomycin

The number of macrophages, neutrophils, and lymphocytes in the airways was examined before (Day 0) and 3, 7, 14, 21, and 28 days after treatment. The total number of leukocytes in the airways before treatment was low, and not different between mice exposed to room air or hyperoxia as neonates (Figure 3A). Treatment with bleomycin resulted in an increase in the number of leukocytes recruited to the lungs in both groups of mice, which peaked on Day 7, and then declined over the next 3 weeks. Neonatal hyperoxia increased the number of leukocytes present in the airspace, and this was significantly greater on Days 7 and 14 after treatment. This reflected a significant increase in macrophages on Day 14, lymphocytes on Days 7 and 14, and neutrophils on Days 3 and 7 (Figures 3B–3D). The marked increase in neutrophils seen on Day 3 is readily apparent in cytopspins (Figure 3E). When calculated as the percentage of the total cell population, neonatal hyperoxia increased the percentage of cells that were neutrophils, while decreasing the percentage of cells that were macrophages (Table 1). This suggests that exposure to neonatal oxygen stimulated the recruitment of leukocytes, and in particular neutrophils, to the lungs of adult mice after treatment with bleomycin.

Anti-Gr-1 Antibody Reduces Oxygen-Mediated Sensitivity to Bleomycin-Induced Lung Fibrosis

Neutrophils were depleted by injecting mice with 300 μ g of anti-Gr-1 antibody. Control animals received rat IgG. This dose of anti-Gr-1 antibody was chosen based upon previous work showing that it effectively depletes neutrophils from the lungs of mice infected with influenza A virus (26). It can also deplete myeloid-derived suppressor cells that control CD8⁺ T-cell responses and, to a lesser extent, CD4⁺ T-cell responses, and promote macrophage differentiation (28). Two hours later, the mice received bleomycin. Compared with neonatal hyperoxia in room air mice, neonatal hyperoxia increased the total number of leukocytes recruited to the lungs of mice treated with bleomycin (Figure 4A). Anti-Gr-1 antibody reduced the total number of leukocytes recruited to the lungs of mice exposed to hyperoxia, but not to room air (Figure 4A). This represented a significant reduction in the number of neutrophils and, to a lesser extent, the number of lymphocytes, but not macrophages (Figures 4B–4D).

To determine whether anti-Gr-1 antibody also attenuated bleomycin-induced lung fibrosis, adult mice exposed to room air or hyperoxia as neonates were injected with anti-Gr-1 or control IgG antibody. Two hours later, they were treated with bleomycin and then injected on Days 3 and 7 after treatment with additional anti-Gr-1 or control IgG antibody. We first confirmed that anti-Gr-1 inhibited hyperoxia-induced neutrophil recruitment on Day 3 by quantifying MPO activity in some mice (Figure 5A). By Day 21 after treatment, soluble collagen concentrations were significantly greater in oxygen-exposed mice treated with bleomycin compared with room air-exposed mice treated with bleomycin (Figure 5B). Although anti-Gr-1 antibody did not affect collagen concentrations in room air-exposed mice treated with bleomycin, it significantly reduced collagen concentrations in oxygen-exposed mice treated with

Figure 2. Neonatal hyperoxia increases bleomycin-induced pulmonary fibrosis. Bleomycin was administered to adult mice that had been exposed to room air (RA) or hyperoxia (O_2) as neonates ($n = 3\text{--}5$ mice per group per time point). (A) Soluble collagen in the left lung lobe was significantly increased in room air-exposed mice on Day 28 ($^aP < 0.0002$) and on Days 21 ($^bP < 0.02$) and 28 ($^cP < 0.002$) in hyperoxia-exposed mice, compared with Day 0 values. Less collagen was detected on Day 21 in room air-exposed mice compared with hyperoxia-exposed mice on Day 14 ($^dP < 0.03$). Columns with the same letter are not significantly different from each other; columns with different letters are significantly different from each other. (B) Representative images of lung tissue stained with trichrome. (C) Gross pathology of the lungs on Day 21 after treatment. Scale bar in B = 100 μm . d, day.

bleomycin. The trichrome staining of lung tissue from these mice confirmed that the previous administration of anti-Gr-1 antibody markedly reduced collagen deposition in oxygen-exposed mice treated with bleomycin (Figure 5C).

TGF- β signaling is required for bleomycin-induced lung fibrosis (29). Quantitative real-time PCR revealed that TGF- β 1

mRNA concentrations were significantly elevated 3 and 7 days after bleomycin treatment in the lung tissue of mice exposed to neonatal hyperoxia, compared with siblings exposed to room air (Figure 6A). The increased expression of TGF- β 1 mRNA correlated with increased concentrations of bioactive TGF- β 1 in BAL fluid (Figure 6A). TGF- β 1 mRNA and protein were then

Figure 3. Neonatal hyperoxia enhances leukocyte recruitment to lungs of adult mice treated with bleomycin. Bleomycin was administered intratracheally to adult mice that had been exposed to room air (RA) or hyperoxia (O_2) as neonates. Bronchoalveolar lavage (BAL) fluid was collected before treatment with bleomycin (Day 0) and on Days 3, 7, 14, 21, and 28 after treatment ($n = 4\text{--}10$ mice per group per time point). The total number of leukocytes (A), macrophages (B), lymphocytes (C), and neutrophils (D) in the BAL fluid of mice was significantly greater in mice exposed to neonatal hyperoxia, compared with mice exposed to room air. ($^*P < 0.02$ for total cell counts, $^*P < 0.05$ for macrophages, $^*P < 0.04$ for lymphocytes, and $^*P < 0.03$ for neutrophils). (E) Representative images of leukocytes collected on Day 7 after treatment.

TABLE 1. DIFFERENTIAL LEUKOCYTE COUNTS IN MICE RECEIVING BLEOMYCIN

Days after Bleomycin	Macrophages (%)		Lymphocytes (%)		Neutrophils (%)	
	RA	O ₂	RA	O ₂	RA	O ₂
Day 0	99 ± 0.3	98 ± 0.4	1 ± 0.7	2 ± 1.1	< 1	< 1
Day 3	58 ± 4.9	28 ± 3.4 [‡]	25 ± 3.2	13 ± 2.7*	17 ± 1.2	60 ± 5.4 [‡]
Day 7	70 ± 3.1	49 ± 3.5*	25 ± 3.1	27 ± 6.1	12 ± 1.1	24 ± 1.0*
Day 14	78 ± 9.0	65 ± 9.6	15 ± 4.4	22 ± 5.1	7 ± 1.2	13 ± 6.1
Day 21	82 ± 1.5	58 ± 2.1 [†]	17 ± 5.4	25 ± 5.3	1 ± 0.6	18 ± 0.7 [‡]
Day 28	82 ± 5.9	58 ± 4.9 [†]	15 ± 3.65.0	26 ± 4.4	3 ± 0.3	18 ± 0.5 [†]

Definition of abbreviations: O₂, hyperoxia; RA, room air.

Adult male mice exposed to room air or hyperoxia as neonates received bleomycin intratracheally. Leukocytes in bronchoalveolar lavage fluid were collected before treatment with bleomycin (Day 0) and on Days 3, 7, 14, 21, and 28 after treatment. Cells were centrifuged onto coded slides and stained with Diff-Quik. The total numbers of macrophages, lymphocytes, and neutrophils were quantified in 200 counted cells. Values represent the mean percentages ± SEMs of macrophages, lymphocytes, and neutrophils ($n = 4-5$ mice per group) in the lavage fluid. Data were analyzed for each cell type by ANOVA with Fisher least significant difference *post hoc* analysis. Neonatal hyperoxia significantly affected the percentages of macrophages, lymphocytes, and neutrophils, compared with room air-exposed mice treated for the same length of time.

* $P < 0.05$.

[†] $P < 0.005$.

[‡] $P < 0.0005$.

quantified in mice treated with control IgG or anti-Gr-1 antibody. On Day 3 after treatment, TGF- β 1 mRNA was significantly increased in hyperoxia-exposed mice treated with control IgG, but was suppressed in hyperoxia-exposed mice treated with anti-Gr-1 antibody, compared with comparably treated room air-exposed mice (Figure 6B). Interestingly, TGF- β 1 mRNA expression increased in room air-exposed mice treated with anti-Gr-1 antibody. Despite increasing TGF- β 1 mRNA, neonatal hyperoxia modestly but not significantly reduced the expression of TGF- β 1. Treatment with anti-Gr-1 antibody did not affect the total amount of TGF- β 1 in BAL fluid (Figure 7B). In contrast, concentrations of active TGF- β 1 increased in mice

exposed to hyperoxia, and this increase was significantly reduced by anti-Gr-1 antibody. Consistent with increased TGF- β 1 activity, neonatal hyperoxia stimulated alveolar phospho-Smad3 staining, and this was reduced by anti-Gr-1 treatment (Figure E2).

Administration of Anti-Gr-1 Antibody after Leukocytes Are Recruited to the Lung Does Not Reduce Hyperoxia-Induced Sensitivity to Bleomycin

To confirm that early leukocyte recruitment was responsible for the hyperoxia-enhanced sensitivity to bleomycin, the anti-Gr-1

Figure 4. Anti-Gr-1 antibody depletes neutrophils and lymphocytes recruited to lungs of hyperoxia-exposed mice treated with bleomycin. Adult male mice exposed to room air (RA) or hyperoxia (O₂) as neonates received anti-Gr-1 antibody, and were then treated with bleomycin 2 hours later. Lungs ($n = 5$ mice per group) were lavaged on Day 3 after treatment. Columns with the same letter are not significantly different from each other; columns with different letters are significantly different from each other. (A) The total number of leukocytes in BAL fluid was significantly greater in mice exposed to hyperoxia, compared with mice exposed to room air ($^bP < 0.003$), but not when mice were treated with anti-Gr-1 antibody. (B) The total number of leukocytes in BAL fluid was not significantly greater in mice exposed to hyperoxia or treated with anti-Gr-1. (C) The total number of lymphocytes in BAL fluid was significantly greater in mice exposed to hyperoxia, compared with mice exposed to room air ($^bP < 0.03$), but not when mice were treated with anti-Gr-1 antibody. (D) The total number of neutrophils in BAL fluid was significantly greater in mice exposed to hyperoxia, compared with mice exposed to room air ($^cP < 0.0001$). The administration of anti-Gr-1 reduced the number of neutrophils recruited to the lungs in mice exposed to room air or hyperoxia ($^bP < 0.009$).

Figure 5. Increased sensitivity to bleomycin-induced lung fibrosis is blocked by anti-Gr-1. Adult male mice exposed to room air (RA) or hyperoxia (O₂) as neonates received anti-Gr-1 antibody and were then treated with bleomycin 2 hours later. Lungs were harvested on Day 3 (A) or Day 21 (B and C) after treatment. Columns with the same letter are not significantly different from each other; columns with different letters are significantly different from each other. (A) Myeloperoxidase (MPO) activity was significantly greater in mice exposed to hyperoxia (^b*P* < 0.02), and was significantly decreased by anti-Gr-1 antibody (^c*P* < 0.02) compared with room air-exposed levels (*n* = 5 mice per group). (B) Soluble collagen was significantly increased on Day 21 after treatment in mice exposed to hyperoxia, compared with mice exposed to room air (^b*P* < 0.001), and was significantly suppressed by anti-Gr-1 antibody (^c*P* < 0.006) compared with room air-exposed mice (*n* = 5–9 mice per group). (C) Representative trichrome-stained images of lung tissue harvested on Day 21 from mice exposed to room air or hyperoxia, with or without previous treatment with anti-Gr-1 antibody. Scale bar = 100 µm.

antibody was administered 3 days after bleomycin treatment. On Day 21 after treatment, soluble collagen concentrations were not different between mice exposed to room air or hyperoxia as neonates (Figure 7A). Trichrome staining confirmed that the administration of anti-Gr-1 antibody after neutrophil recruitment did not diminish hyperoxia-induced lung fibrosis (Figure 7B).

DISCUSSION

Growing evidence indicates that prenatal and postnatal environmental factors affect the normal structural development and programming of the lung and immune system. Environmental stressors that adversely affect lung structure and function include hyperoxia, preterm birth, infection, tobacco smoke, and exposure to various chemicals or drugs (30–32). However, the mechanisms by which early-life environmental stressors disrupt lung and immune function are presently not known, which hampers our ability to identify early disease risk markers and clinical outcomes based on disease mechanism, and to design early interventions to prevent or cure lung disease. In the present study, we provide evidence that early-life exposure to hyperoxia enhances the profibrotic effects of bleomycin in adult mice. Increased sensitivity to bleomycin-induced lung fibrosis was attributed to the rapid recruitment of leukocytes, in particular neutrophils, and elevated TGF-β1 in BAL fluid. Interestingly, the effects on neutrophil recruitment were more pronounced than when hyperoxia-exposed mice were infected with a sublethal dose of influenza A virus (HKx31, H3N2), wherein the increased percentage of neutrophils was similar to that of macrophages and lymphocytes (21). Subsequent studies revealed that neonatal hyperoxia did not impair the cytotoxic effects of CD8⁺ T cells or memory T cells (33). Instead, neonatal hyperoxia reduced the epithelial expression of eosinophil-associated RNase (Ear)1, an antiviral RNase that inhibits viral replication,

and this was associated with greater epithelial injury (34). Taken together, these findings suggest that neonatal hyperoxia promotes fibrotic disease by increasing epithelial injury, which in the case of bleomycin could be enhanced by an influx of leukocytes, principally neutrophils, and a rapid activation of TGF-β1.

Patients with idiopathic pulmonary fibrosis (IPF) present with breathlessness on exertion, dry cough, subpleural honeycombing on high-resolution computed tomography, and the presence of hyperplastic epithelial cells overlying organized myofibroblasts in biopsies (35). Because these lesions are often patchy, lacking inflammation, and refractory to conventional anti-inflammatory strategies, the concept that IPF is an inflammatory-mediated lung disease has now shifted to considering it a disease of repetitive epithelial injury with impaired repair. Despite the overt lack of inflammation in fibrosis, inflammation clearly contributes to disease progression, as reviewed by Coward and colleagues (36). The recruitment of granulocytes, monocytes, and circulating fibrocytes after injury is thought to be beneficial because they release factors that enhance the expression of the vascular endothelial growth factor, keratinocyte growth factor, and TGF-β1 required for appropriate repairs of the injured lung. However, too many leukocytes or the recruitment of overly activated leukocytes can cause excessive epithelial injury, overwhelm repairs, and skew tissue homeostasis toward disease. We found that neonatal hyperoxia enhanced the recruitment of leukocytes and in particular neutrophils to the lungs of adult mice receiving bleomycin. Neutrophils have been detected in the lung parenchyma of patients with IPF, and are a rich source of matrix-degrading enzymes, including neutrophil elastase (37). Mice lacking neutrophil elastase are resistant to bleomycin-induced lung fibrosis (38). Because neutrophil elastase can digest most matrix proteins, it may potentiate epithelial injury and the release of profibrotic molecules such as TGF-β1 from the matrix (39). Neutrophils are also a rich source of active

Figure 6. Pretreatment with anti-Gr-1 antibody inhibits the activation of transforming growth factor (TGF)-β1 in hyperoxia-exposed mice treated with bleomycin. Columns with the same letter are not significantly different from each other; columns with different letters are significantly different from each other. (A) Bleomycin was administered intratracheally to adult mice that had been exposed to room air (RA) or hyperoxia (O₂) as neonates ($n = 3$ mice per group). TGF-β1 mRNA ($^bP < 0.01$), active TGF-β1 in BAL fluid ($^bP < 0.0001$ on Day 3, and $^cP < 0.001$ on Day 7), and total TGF-β1 ($^cP < 0.01$) significantly increased in mice treated with bleomycin. (B) Control IgG or anti-Gr-1 antibody was injected into young adult mice exposed to room air or hyperoxia as neonates. Mice were then treated with bleomycin and killed 3 days later ($n = 4-5$ mice per group). TGF-β1 mRNA significantly increased ($^bP < 0.02$), but was significantly reduced by anti-Gr-1 antibody ($^cP < 0.003$). Active TGF-β1 significantly increased ($^bP < 0.05$), but was reduced by anti-Gr-1 antibody ($^cP < 0.01$). Total concentrations of TGF-β1 protein were not affected by neonatal hyperoxia or by anti-Gr-1 antibody on Day 3 after treatment ($^aP = 0.2$).

TGF-β1 (40). Although neonatal hyperoxia increases TGF-β1 expression, and the depletion of neutrophils using the anti-Gr-1 antibody reduced the expression of TGF-β1, it remains to be determined whether neutrophils are the primary source of TGF-β1 expression. Surprisingly, anti-Gr-1 antibody did not affect bleomycin-induced lung fibrosis or MPO activity in room air-exposed mice, perhaps because some neutrophils are needed for a normal response to injury, or perhaps because murine monocytes can also be a source of MPO activity.

Alternatively, epithelial injury occurring in room air mice may have been caused by a low dose of bleomycin, whereas epithelial injury occurring in hyperoxia-exposed mice was caused by the low dose of bleomycin and the recruitment of inflammatory cells such as neutrophils. Thus, the identification of neutrophil chemoattractants produced in adult mice exposed to neonatal hyperoxia and treated with bleomycin should help us better understand how neonatal hyperoxia reprograms the response of the lung to epithelial injury.

Figure 7. Administration of anti-Gr-1 antibody after TGF-β1 is activated does not alleviate hyperoxia-induced lung fibrosis. Adult male mice exposed to room air (RA) or hyperoxia (O₂) as neonates received bleomycin intratracheally. Mice then received control IgG or anti-Gr-1 antibody on Days 3 and 7 after treatment. Columns with the same letter are not significantly different from each other; columns with different letters are significantly different from each other. (A) Bleomycin significantly increased soluble collagen in oxygen-exposed mice treated with IgG or anti-Gr-1 antibody ($^bP < 0.05$), compared with room air-exposed mice. (B) Representative images of trichrome-stained lung tissue from room air-exposed or oxygen-exposed mice, harvested 21 days after treatment with bleomycin. Scale bar = 100 μm.

The early activation of TGF- β 1 likely constitutes one way by which neonatal hyperoxia enhances bleomycin-induced lung fibrosis. Bleomycin-induced lung fibrosis is dependent upon TGF- β signaling through Smad3 (29). The overexpression of biologically activated TGF- β 1 also causes fibrosis, and this fibrosis can be alleviated by the loss of Smad3 (41, 42). TGF- β signaling can drive fibrosis through multiple mechanisms, including prevention of the death of activated T lymphocytes, hence permitting the survival of immune cells that may contribute to inflammation and epithelial injury (43). TGF- β signaling can also stimulate the proliferation of fibroblasts (44), inhibit myofibroblast apoptosis (45), stimulate matrix deposition (46), and promote the epithelial-mesenchymal transition (47, 48). Because TGF- β regulates so many biologic processes, to identify a singular mechanism by which it promotes fibrosis has been difficult. The knowledge that the adenoviral-mediated delivery of active TGF- β 1 promotes fibrosis in “fibrosis-prone” C57Bl/6 mice to a lesser extent than in “fibrosis-resistant” BALB/c mice clearly indicates that other downstream modifiers modulate the fibrotic actions of TGF- β (49). The extent of epithelial injury may be one of the modifiers.

TGF- β signaling may also be modified by how well the epithelium responds to injury. Emerging evidence suggests that repetitive epithelial injury contributes to IPF (35). Repetitive epithelial injury, as caused by the combination of neonatal hyperoxia and influenza A virus or neonatal hyperoxia and bleomycin, may be a common feature in our two models. Perhaps repetitive injury reprograms whether the epithelium participates in repair or undergoes EMT. Evidence supporting the existence of an EMT derives from studies showing how TGF- β can stimulate primary cultures of Type II cells or various epithelial cell lines to take on a mesenchymal phenotype (47, 50, 51). However, fate-mapping studies with lineage-labeled mice have raised questions about whether the EMT actually exists *in vivo* (47, 48, 51, 52). We did not see significant evidence of EMT in mice treated with bleomycin, regardless of whether they were exposed to room air or hyperoxia as neonates. Our findings are consistent with recent studies suggesting that EMT plays a minor role, if it occurs at all, in pulmonary fibrosis (48, 52). However, some epithelial cells that contribute to EMT may not have been durably labeled using this approach. Alternatively, neonatal hyperoxia might affect the progenitor cells needed to repair injured epithelium. Indeed, adult mice exposed to neonatal hyperoxia demonstrate fewer epithelial Type II cells (20). The loss of Type II cells in young adult mice exposed to neonatal hyperoxia may also contribute to fibrosis, because Type II cells expand and differentiate into Type I cells after alveolar epithelial injury (53). Cultured Type II cells also secrete IL-1 α , which stimulates the fibroblast production of prostaglandin E2, an inhibitor of fibroblast growth (54, 55). Similarly, the conditional ablation of Type II cells expressing the diphtheria toxin receptor spontaneously results in mild fibrosis (56). Hence, lower numbers of Type II cells in mice exposed to neonatal hyperoxia could allow for greater fibroblast expansion when the epithelium is damaged by viral infection or bleomycin.

In conclusion, the present study shows that neonatal hyperoxia enhances the fibrotic properties of bleomycin by stimulating leukocyte recruitment and the accumulation of bioactive TGF- β 1 in the lung. It extends previous work showing that neonatal hyperoxia promotes fibrotic disease after infection with a sublethal dose of influenza A virus (21, 22, 33, 34). Our findings suggest that individuals born prematurely and exposed to hyperoxia may be at increased risk for developing pulmonary fibrosis when their respiratory epithelium is damaged later in life.

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References

- Coalson JJ. Pathology of bronchopulmonary dysplasia. *Semin Perinatol* 2006;30:179–184.
- Jobe AH, Kallapur SG. Long term consequences of oxygen therapy in the neonatal period. *Semin Fetal Neonatal Med* 2010;15:230–235.
- Smith VC, Zupancic JA, McCormick MC, Croen LA, Greene J, Escobar GJ, Richardson DK. Rehospitalization in the first year of life among infants with bronchopulmonary dysplasia. *J Pediatr* 2004;144:799–803.
- Doyle LW, Faber B, Callanan C, Freezer N, Ford GW, Davis NM. Bronchopulmonary dysplasia in very low birth weight subjects and lung function in late adolescence. *Pediatrics* 2006;118:108–113.
- Robin B, Kim YJ, Huth J, Klocksieben J, Torres M, Tepper RS, Castile RG, Solway J, Hershenson MB, Goldstein-Filbrun A. Pulmonary function in bronchopulmonary dysplasia. *Pediatr Pulmonol* 2004;37:236–242.
- Groothuis JR, Gutierrez KM, Lauer BA. Respiratory syncytial virus infection in children with bronchopulmonary dysplasia. *Pediatrics* 1988;82:199–203.
- Cunningham CK, McMillan JA, Gross SJ. Rehospitalization for respiratory illness in infants of less than 32 weeks' gestation. *Pediatrics* 1991;88:527–532.
- Delobel-Ayoub M, Arnaud C, White-Koning M, Casper C, Pierrat V, Garel M, Burguet A, Roze JC, Matis J, Picaud JC, *et al.* Behavioral problems and cognitive performance at 5 years of age after very preterm birth: the EPIPAGE Study. *Pediatrics* 2009;123:1485–1492.
- Woythaler MA, McCormick MC, Smith VC. Late preterm infants have worse 24-month neurodevelopmental outcomes than term infants. *Pediatrics* 2011;127:e622–e629.
- Chen Y, Frank L. Differential gene expression of antioxidant enzymes in the perinatal rat lung. *Pediatr Res* 1993;34:27–31.
- Clerch LB, Massaro D. Rat lung antioxidant enzymes: differences in perinatal gene expression and regulation. *Am J Physiol* 1992;263:L466–L470.
- Kennedy JD, Edward LJ, Bates DJ, Martin AJ, Dip SN, Haslam RR, McPhee AJ, Staugas RE, Baghurst P. Effects of birthweight and oxygen supplementation on lung function in late childhood in children of very low birth weight. *Pediatr Pulmonol* 2000;30:32–40.
- Coates AL, Desmond K, Willis D, Nogrady MB. Oxygen therapy and long-term pulmonary outcome of respiratory distress syndrome in newborns. *Am J Dis Child* 1982;136:892–895.
- Davis JM, Parad RB, Michele T, Allred E, Price A, Rosenfeld W. Pulmonary outcome at 1 year corrected age in premature infants treated at birth with recombinant human CuZn superoxide dismutase. *Pediatrics* 2003;111:469–476.
- Coalson JJ, Winter VT, Siler-Khodr T, Yoder BA. Neonatal chronic lung disease in extremely immature baboons. *Am J Respir Crit Care Med* 1999;160:1333–1346.
- Warner BB, Stuart LA, Papes RA, Wispe JR. Functional and pathological effects of prolonged hyperoxia in neonatal mice. *Am J Physiol* 1998;275:L110–L117.
- Pillow JJ, Hillman NH, Polglase GR, Moss TJ, Kallapur SG, Cheah FC, Kramer BW, Jobe AH. Oxygen, temperature and humidity of inspired gases and their influences on airway and lung tissue in near-term lambs. *Intensive Care Med* 2009;35:2157–2163.
- Dauger S, Ferkdadi L, Saumon G, Vardon G, Peuchmaur M, Gaultier C, Gallego J. Neonatal exposure to 65% oxygen durably impairs lung architecture and breathing pattern in adult mice. *Chest* 2003;123:530–538.
- McGrath-Morrow SA, Cho C, Soutiere S, Mitzner W, Tudor R. The effect of neonatal hyperoxia on the lung of p21WAF1/CIP1/SDI1-deficient mice. *Am J Respir Cell Mol Biol* 2004;30:635–640.
- Yee M, Vitiello PF, Roper JM, Staversky RJ, Wright TW, McGrath-Morrow SA, Maniscalco WM, Finkelstein JN, O'Reilly MA. Type II epithelial cells are critical target for hyperoxia-mediated impairment

- of postnatal lung development. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L1101–L1111.
21. O'Reilly MA, Marr SH, Yee M, McGrath-Morrow SA, Lawrence BP. Neonatal hyperoxia enhances the inflammatory response in adult mice infected with influenza A virus. *Am J Respir Crit Care Med* 2008; 177:1103–1110.
22. Buczynski BW, Yee M, Paige Lawrence B, O'Reilly MA. Lung development and the host response to influenza A virus are altered by different doses of neonatal oxygen in mice. *Am J Physiol Lung Cell Mol Physiol* 2012;302:L1078–L1087.
23. Adamson IY, Bowden DH. The pathogenesis of bleomycin-induced pulmonary fibrosis in mice. *Am J Pathol* 1974;77:185–197.
24. Walters DM, Kleeberger SR. Mouse models of bleomycin-induced pulmonary fibrosis. *Curr Protoc Pharmacol* 2008;40:5.46.1–5.46.17.
25. Adamson IY, Bowden DH. Bleomycin-induced injury and metaplasia of alveolar Type 2 cells: relationship of cellular responses to drug presence in the lung. *Am J Pathol* 1979;96:531–544.
26. Teske S, Bohn AA, Regal JF, Neumiller JJ, Lawrence BP. Activation of the aryl hydrocarbon receptor increases pulmonary neutrophilia and diminishes host resistance to influenza A virus. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L111–L124.
27. Yee M, White RJ, Awad HA, Bates WA, McGrath-Morrow SA, O'Reilly MA. Neonatal hyperoxia causes pulmonary vascular disease and shortens life span in aging mice. *Am J Pathol* 2011;178:2601–2610.
28. Ribechini E, Leenen PJ, Lutz MB. Gr-1 antibody induces STAT signaling, macrophage marker expression and abrogation of myeloid-derived suppressor cell activity in BM cells. *Eur J Immunol* 2009;39: 3538–3551.
29. Zhao J, Shi W, Wang YL, Chen H, Bringas P Jr, Datto MB, Frederick JP, Wang XF, Warburton D. Smad3 deficiency attenuates bleomycin-induced pulmonary fibrosis in mice. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L585–L593.
30. Plopper CG, Smiley-Jewell SM, Miller LA, Fanucchi MV, Evans MJ, Buckpitt AR, Avdalovic M, Gershwin LJ, Joad JP, Kajekar R, et al. Asthma/allergic airways disease: does postnatal exposure to environmental toxicants promote airway pathobiology? *Toxicol Pathol* 2007;35:97–110.
31. Pinkerton KE, Joad JP. The mammalian respiratory system and critical windows of exposure for children's health. *Environ Health Perspect* 2000;108:457–462.
32. Kajekar R. Environmental factors and developmental outcomes in the lung. *Pharmacol Ther* 2007;114:129–145.
33. Giannandrea M, Yee M, O'Reilly MA, Lawrence BP. Memory CD8⁺ T cells are sufficient to alleviate impaired host resistance to influenza A virus infection caused by neonatal oxygen supplementation. *Clin Vaccine Immunol* 2012;19:1432–1441.
34. O'Reilly MA, Yee M, Buczynski BW, Vitiello PF, Keng PC, Welle SL, Finkelstein JN, Dean DA, Lawrence BP. Neonatal oxygen increases sensitivity to influenza A virus infection in adult mice by suppressing epithelial expression of Ear1. *Am J Pathol* 2012;181:441–451.
35. Zoz DF, Lawson WE, Blackwell TS. Idiopathic pulmonary fibrosis: a disorder of epithelial cell dysfunction. *Am J Med Sci* 2011;341:435–438.
36. Coward WR, Saini G, Jenkins G. The pathogenesis of idiopathic pulmonary fibrosis. *Ther Adv Respir Dis* 2010;4:367–388.
37. Obayashi Y, Yamadori I, Fujita J, Yoshinouchi T, Ueda N, Takahara J. The role of neutrophils in the pathogenesis of idiopathic pulmonary fibrosis. *Chest* 1997;112:1338–1343.
38. Chua F, Dunsmore SE, Clingen PH, Mutsaers SE, Shapiro SD, Segal AW, Roes J, Laurent GJ. Mice lacking neutrophil elastase are resistant to bleomycin-induced pulmonary fibrosis. *Am J Pathol* 2007;170: 65–74.
39. Taipale J, Lohi J, Saarinen J, Kovanen PT, Keski-Oja J. Human mast cell chymase and leukocyte elastase release latent transforming growth factor- β 1 from the extracellular matrix of cultured human epithelial and endothelial cells. *J Biol Chem* 1995;270:4689–4696.
40. Grotendorst GR, Smale G, Pencev D. Production of transforming growth factor β by human peripheral blood monocytes and neutrophils. *J Cell Physiol* 1989;140:396–402.
41. Bonniaud P, Kolb M, Galt T, Robertson J, Robbins C, Stampfli M, Lavery C, Margetts PJ, Roberts AB, Gauldie J. Smad3 null mice develop airspace enlargement and are resistant to TGF- β -mediated pulmonary fibrosis. *J Immunol* 2004;173:2099–2108.
42. Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J. Adenovector-mediated gene transfer of active transforming growth factor- β 1 induces prolonged severe fibrosis in rat lung. *J Clin Invest* 1997;100: 768–776.
43. Wahl SM, Orenstein JM, Chen W. TGF- β influences the life and death decisions of T lymphocytes. *Cytokine Growth Factor Rev* 2000; 11:71–79.
44. Dkhissi F, Raynal S, Jullien P, Lawrence DA. Growth stimulation of murine fibroblasts by TGF- β 1 depends on the expression of a functional p53 protein. *Oncogene* 1999;18:703–711.
45. Hashimoto N, Jin H, Liu T, Chensue SW, Phan SH. Bone marrow-derived progenitor cells in pulmonary fibrosis. *J Clin Invest* 2004;113: 243–252.
46. Broekelmann TJ, Limper AH, Colby TV, McDonald JA. Transforming growth factor β 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. *Proc Natl Acad Sci USA* 1991;88:6642–6646.
47. Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, du Bois RM, Borok Z. Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor- β 1: potential role in idiopathic pulmonary fibrosis. *Am J Pathol* 2005;166:1321–1332.
48. Tanjore H, Xu XC, Polosukhin VV, Degryse AL, Li B, Han W, Sherrill TP, Plieth D, Neilson EG, Blackwell TS, et al. Contribution of epithelial-derived fibroblasts to bleomycin-induced lung fibrosis. *Am J Respir Crit Care Med* 2009;180:657–665.
49. Kolb M, Bonniaud P, Galt T, Sime PJ, Kelly MM, Margetts PJ, Gauldie J. Differences in the fibrogenic response after transfer of active transforming growth factor- β 1 gene to lungs of “fibrosis-prone” and “fibrosis-resistant” mouse strains. *Am J Respir Cell Mol Biol* 2002;27:141–150.
50. Kasai H, Allen JT, Mason RM, Kamimura T, Zhang Z. TGF- β 1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respir Res* 2005;6:56.
51. Kim KK, Kugler MC, Wolters PJ, Robillard L, Galvez MG, Brumwell AN, Sheppard D, Chapman HA. Alveolar epithelial cell mesenchymal transition develops *in vivo* during pulmonary fibrosis and is regulated by the extracellular matrix. *Proc Natl Acad Sci USA* 2006;103: 13180–13185.
52. Rock JR, Barkauskas CE, Cronic MJ, Xue Y, Harris JR, Liang J, Noble PW, Hogan BL. Multiple stromal populations contribute to pulmonary fibrosis without evidence for epithelial to mesenchymal transition. *Proc Natl Acad Sci USA* 2011;108:E1475–E1483.
53. Fehrenbach H. Alveolar epithelial Type II cell: defender of the alveolus revisited. *Respir Res* 2001;2:33–46.
54. Pan T, Mason RJ, Westcott JY, Shannon JM. Rat alveolar Type II cells inhibit lung fibroblast proliferation *in vitro*. *Am J Respir Cell Mol Biol* 2001;25:353–361.
55. Portnoy J, Pan T, Dinarello CA, Shannon JM, Westcott JY, Zhang L, Mason RJ. Alveolar Type II cells inhibit fibroblast proliferation: role of IL-1 α . *Am J Physiol Lung Cell Mol Physiol* 2006;290:L307–L316.
56. Sisson TH, Mendez M, Choi K, Subbotina N, Courey A, Cunningham A, Dave A, Engelhardt JF, Liu X, White ES, et al. Targeted injury of Type II alveolar epithelial cells induces pulmonary fibrosis. *Am J Respir Crit Care Med* 2010;181:254–263.