Animal Model

Transfer of the Active Form of Transforming Growth Factor- β 1 Gene to Newborn Rat Lung Induces Changes Consistent with Bronchopulmonary Dysplasia

Jack Gauldie,* Tom Galt,* Philippe Bonniaud,*[†]
Clinton Robbins,* Margaret Kelly,* and
David Warburton[‡]

From the Department of Pathology and Molecular Medicine,*
McMaster University, Hamilton, Canada; the Service de
Pneumologie et Réanimation Respiratoire,[†] Centre Hospitalo-
Universitaire du Bocage et Université de Bourgogne, Dijon,
France; and the Developmental Biology Program,[‡] Children's
Hospital Los Angeles Research Institute, Keck School of Medicine,
University of Southern California, Los Angeles, California

Bronchopulmonary dysplasia is a chronic lung disease of premature human infancy that shows pathological features comprising varying sized areas of interstitial fibrosis in association with distorted large alveolar spaces. We have previously shown that transfer of active transforming growth factor (TGF)- β 1 (AdTGF β 1^{223/225}) genes by adenovirus vector to embryonic lungs results in inhibition of branching morphogenesis and primitive peripheral lung development, whereas transfer to adult lungs results in progressive interstitial fibrosis. Herein we show that transfer of TGF- β 1 to newborn rat pups results in patchy areas of interstitial fibrosis developing throughout a period of 28 days after transfer. These areas of fibrosis appear alongside areas of enlarged alveolar spaces similar to the prealveoli seen at birth, suggesting that postnatal lung development and alveolarization has been inhibited. In rats treated with AdTGF β 1^{223/225}, enlarged alveolar spaces were evident by day 21, and by 28 days, the mean alveolar cord length was nearly twice that in control vector or untreated rats. Hydroxyproline measurements confirmed the presence of fibrosis. These data suggest that overexpression of TGF- β 1 during the critical period of postnatal rat lung alveolarization gives rise to pathological, biochemical, and morphological changes consistent with those seen in human bronchopulmonary dysplasia, thus inferring a pathogenic

role for TGF- β in this disorder. (Am J Pathol 2003, 163:2575–2584)

Bronchopulmonary dysplasia (BPD) remains an all too common complication of human premature delivery, despite the advent of artificial surfactants, antenatal and postnatal treatment with glucocorticoids, and the increasing use of gentler modes of mechanical ventilation.^{1,2} The term BPD was coined to describe a radiographical entity, comprising patchy interstitial fibrosis along with distortion of peripheral air spaces, which occurs after ventilation of babies with hyaline membrane disease.³ This pathology was thought to be a sequela of abnormal repair processes after the resolution of hyaline membrane disease of the newborn. BPD is now more frequently diagnosed in very premature human infants with chronic lung disease who require long-term oxygen therapy.⁴ BPD is thought to begin as postinjury inflammation, leading to interstitial fibrosis. But, as the lung continues to enlarge, reduced numbers of enlarged distal air spaces also appear.⁵ The pathogenesis of this disorder is poorly understood despite numerous studies. With the advent of exogenous pulmonary surfactant and more frequent glucocorticoid therapy, the incidence of this form of BPD has fortunately decreased. However, among extremely low birth weight infants, new BPD has emerged as an increasingly common problem.^{1,2,6} The pathology of the new BPD is characterized primarily by alveolar hypoplasia with some interstitial cellular infiltrates and less marked interstitial fibrosis, and has been considered an arrest of lung development.⁴ However, recent data showing involvement

Supported by the Canadian Institutes of Health Research, Hamilton Health Sciences, St. Joseph's Healthcare, and the National Institutes of Health (National Heart, Lung, and Blood Institute grant PO1HL60231).

Accepted for publication August 27, 2003.

Address reprint requests to Dr. Jack Gauldie, Department of Pathology and Molecular Medicine 2N16, McMaster University, 1200 Main St. West, Hamilton, Ontario, Canada L8N 3Z5. E-mail: gauldie@mcmaster.ca.

of growth and repair factors such as transforming growth factor (TGF)- β in adult pulmonary fibrosis,⁷⁻⁹ as well as the role of TGF- β as an inhibitor of branching morphogenesis in embryonic lung development,¹⁰⁻¹² suggested to us that factors such as TGF- β may induce similar changes in neonatal lung.

We have previously used adenovirus vectors to effect transient gene transfer of active TGF- β 1 (AdTGF β 1^{223/225}) to the lung of adult rats⁹ resulting in the development of progressive interstitial fibrosis. Herein we have used the same vector to transfer TGF- β 1 to the lung of 1-day-old rat pups. We show that this transient overexpression (5 to 10 days) of TGF- β 1 causes morphological and biochemical changes consistent with interstitial fibrosis along with large distorted alveoli that appear within a few weeks after birth, pathological features strikingly similar to those seen in human BPD.

Materials and Methods

Administration of Adenovector to Neonatal Rat Lung

One-day-old neonatal Sprague-Dawley rats were exposed to isoflurane for 30 to 40 seconds to provide light anesthesia and were administered intranasally with 1×10^8 plaque forming units (PFUs) of either Ad dl70-3 (control vector, no transgene) or AdTGF β 1^{223/225} (vector expressing the active form of TGF- β 1) diluted in 10 μ l of sterile phosphate-buffered saline (PBS). Holding the anesthetized rat upright, the adenoviral solution was placed into the nasal passages using a P10 pipette. Using the thumb, slight pressure was placed on the bottom of the jaw to keep the mouth closed and force the animal to inhale the solution through its nose. The rats were held upright for a further 20 to 30 seconds to maximize delivery, and then allowed to recover under supervision before returning them to their mother. Preliminary studies with AdLacZ (vector expressing LacZ) showed this approach gave rise to wide distribution of the vector throughout the lung with uptake in bronchial and bronchiolar epithelial cells, similar to the distribution seen in adult rats with either intranasal or intratracheal instillation (data not shown). The control vector (empty) was chosen to avoid any confusing issues resulting from known immune responses against an expressed foreign transgene, such as LacZ.¹³ The TGF- β 1 gene is highly homologous and does not engender immune reactions in the host.

Detection of Transgene Expression

Because of the small and delicate nature of neonatal rat lungs, bronchoalveolar lavage could not readily be performed in a consistent manner to assess the presence of TGF- β 1 transgene. To accomplish this, three to five neonatal rats per group were sacrificed 2 days after adenovector injection. Before excising their lungs, the right ventricle of the heart was perfused with 3 ml of ice-cold PBS to remove all blood from the lungs. The lungs were

then excised, weighed, and kept in ice-cold PBS. Lung weights did not vary significantly between individual rats or treated groups throughout the extent of the experiment. Shortly thereafter, lungs were transferred to 2 ml of fresh PBS and homogenized. The homogenate was pelleted by centrifugation at 12,000 rpm for 10 minutes at 4°C and the cytokine-rich supernatant was stored at -70°C until assayed. TGF- β 1 levels were determined as described below and expressed as pg of active TGF- β 1 per mg wet lung weight.

TGF- β 1 Assay

TGF- β 1 levels were determined using a human TGF- β 1 enzyme-linked immunosorbent assay kit (R&D Systems, Inc., Minneapolis, MN) which is totally cross-reactive with porcine and rodent TGF- β 1, but does not cross-react with TGF- β 2 or TGF- β 3. Total TGF- β 1 levels were measured by acid-activating samples as per the manufacturer's protocol. Levels of active TGF- β 1 were measured by assaying samples that were not acid-activated.

Lung Hydroxyproline Determination

A modified Woessner protocol¹⁴ was used. Tissue was prepared by immediately snap-freezing the right lobe of excised lungs in liquid nitrogen, then transferring the samples to -70°C for storage until the time of assay. Samples were homogenized in 5 ml of ddH₂O. One ml of the resultant homogenate was hydrolyzed in 2 ml of 6 N HCl for 16 hours at 110°C. Samples were adjusted to pH 7.0 and then 400 μ l was diluted into 2.0 ml with ddH₂O. The colorimetric assay was initiated with the addition of 1 ml of chloramine-T solution to the diluted sample at pH 7.0. One ml of 70% perchloric acid was added, followed by 1 ml of a dimethylbenzaldehyde solution. After 20 minutes at 60°C, the samples were returned to room temperature and the optical density at 557 nm was determined. Using hydroxyproline standards (Sigma Chemical, St. Louis, MO), results were expressed as μ g hydroxyproline per mg wet lung weight.

Lung Fixation and Histological Examination

In adult mice, on lung excision, the left lobe was inflated and perfused with 10% formalin, and fixed for 24 hours before processing and paraffin embedding. In neonates, <10 days old, tissue was not perfused before fixation because of the small size of vessel for cannulation. Histological sections were stained with hematoxylin and eosin, a general nuclear stain, and elastin van Gieson, which stains collagen and elastin. Sections were also stained for collagen matrix with Sirius Red F3B (CI 35780) (Sigma Aldrich Can. Ltd., Oakville, Ontario, Canada) in saturated picric acid (Picrosirius Red stain). Select sections also underwent immunohistochemical staining for α -smooth muscle actin (α -SMA), as previously described.⁹

Determination of Average Alveolar Size

The size of alveolar space was determined from lung sections stained with Picrosirius Red. Mean chord length (μm) was measured at $\times 100$ magnification and is calculated from automated morphometric analysis using a Leica Q500IW high-grade image analysis system. Chord length increases with alveolar enlargement and is similar to mean linear intercept with the advantage of being independent of the thickness of septa.¹⁵

Data Analysis

Data were expressed as mean $\pm$ SEM or SD. Statistical significance was determined using Student's two-tailed *t*-test, assuming unequal variances. Differences were considered statistically significant if *P* was < 0.05 . All pairwise multiple comparisons were subjected to the Tukey test. In considering comparisons between three groups of animals, a one-way analysis of variance test was performed.

Results

Administering 1-day-old rat pups intranasally with adenovector is an inherently difficult task because of their frailty and small size. The efficacy of the intranasal administration method was therefore assessed by measuring the amount of bioactive TGF- β 1 within the lungs of pups 2 days after infection, comparing the control adenovector with the active TGF- β 1-expressing adenovector. Although bronchoalveolar lavage fluid is generally examined to identify cytokines important in the lung microenvironment, the friable nature of the neonatal rat trachea prevented accurate bronchoalveolar lavage fluid recovery. As an alternative, lungs from 3-day-old pups (2 days after administration) were excised, perfused free of blood using ice-cold PBS, and quickly homogenized. After brief centrifugation, supernatants were examined using enzyme-linked immunosorbent assay (Figure 1). Expressed as pg of TGF- β 1 per mg of lung tissue, the pups administered AD5E1TGF β 1^{223/225} showed significantly higher (>30 -fold) levels of total and active (10-fold) TGF- β 1 than those treated with control vector ($P < 0.02$). There was no significant difference in TGF- β 1 levels between control vector-treated and untreated groups.

At 7 days after infection (8 days old) normal rat lung showed a hypercellular thick interstitium, made up primarily from noninflammatory cells (Figure 2A) and alveoli that appeared to be smaller in size than those seen at birth. The animals that were treated with the control vector, dL70-3, showed a similar hypercellular interstitium with evidence of some inflammatory cells, neutrophils, and mononuclear cells (Figure 2B). The animals that were treated with AdTGF- β 1 also showed a hypercellular interstitium with a number of inflammatory cells, along with areas of apparent consolidation (Figure 2C), seen to be positive for matrix deposition by staining with Picrosirius Red (Figure 2F).

Figure 1. TGF- β 1 levels in lung homogenate of 3-day-old rat, untreated, or 2 days after intranasal administration of 1×10^8 PFU AdDL70 or AdTGF β 1^{223/225}. Levels of total TGF- β 1 (white bars) and active TGF- β 1 (black bars), as measured by enzyme-linked immunosorbent assay, are expressed as pg of TGF- β 1 per mg of lung. Significant differences were seen between the TGF- β 1 vector-treated group and the control groups for levels of total TGF- β 1 (*, $P < 0.02$) and active TGF- β 1 (#, $P < 0.02$; $n = 3$ to 5 per group). There were no significant differences between the control vector group and normal untreated animals.

Throughout a period of days, the presence of inflammatory cells decreased in the two vector-treated groups, and the alveolar septa decreased in thickness in all three groups and by 29 days (28 days after injection) the lungs of the control vector group demonstrated fundamentally normal lung architecture when compared to untreated lung (Figure 3). Alveoli were divided and subdivided into their saccular network, providing a much larger area for gas exchange than was available at birth when the alveoli had not yet fully compartmentalized. Rat pups injected with the TGF- β 1-expressing adenovirus vector developed disperse fibrotic lesions within their lungs (Figure 4), although not to the extent demonstrated in experiments in which the same vector was administered intratracheally to adult rats.⁹ There was marked deposition of matrix within the parenchyma localized to defined areas throughout the lung. These were not particularly distributed, for example, in peribronchial areas, but tended to be associated at air interfaces rather than at vascular sites. Analysis of hydroxyproline content at 29 days showed an increase in matrix accumulation within the lungs of the Ad5E1TGF β 1^{223/225}-treated pups compared to the control vector group (Figure 5). Using a one-way analysis of variance, the differences between the three groups showed $P = 0.078$. Using an independent *t*-test, day 28 TGF- β 1-treated animals differed from day 21 TGF- β 1-treated animals ($P < 0.05$) and day 28 TGF- β 1-treated animals differed from control vector-treated animals ($P < 0.05$). There were no significant differences seen at 21 days, consistent with the progressive nature of fibrosis initiated by TGF- β 1 overexpression in adult rats.⁹ The areas positive for matrix deposition were also prominent for α -SMA-positive cells, most likely myofibroblasts. In addition, there were prominent α -SMA-positive cells in the alveolar walls indicating an enhanced presence of myofibroblasts throughout the lung parenchyma (Figure 6).

Figure 2. Eight-day-old neonatal rat lung (7 days after treatment). **A, D:** Control lung, untreated. **B, E:** Lung treated with AdDL70-3. **C, F:** Lung treated with AdTGF β 1^{223/225}. **A, B,** and **C** were stained with H&E and **D, E,** and **F** were stained with Picrosirius Red. Original magnifications, $\times 200$.

Although excess matrix deposition (fibrosis) is a prominent feature in the Ad5E1TGF β 1^{223/225}-treated lungs, even more striking are the large, apparently undeveloped prealveolar saccules that predominate in the lungs of pups from this group (Figure 6). There were no significant differences in alveolar space sizes at birth between the treatment groups (Table 1). By 21 days, the enlarged

spaces were clearly evident with $\sim 50\%$ increase in mean alveolar chord length from control-treated mice, and at 28 days, the increase was nearly 80% ($P > 0.001$ for both comparisons against untreated or control vector-treated animals) indicating the presence of markedly enlarged alveoli throughout the lung. The areas of enlarged alveoli are in many instances immediately adjacent to areas

Figure 3. Twenty-nine-day-old neonatal rat lung (28 days after treatment). **A, B:** Untreated. **C, D:** Treatment with control vector Ad dl70-3. **A** and **C** were stained with H&E and **B** and **D** were stained with Picrosirius Red. Lungs of both groups appear histologically normal. Original magnifications, $\times 100$.

showing evidence of marked fibrosis (Figure 7) suggestive of pathology seen in human BPD.⁵ In isolated cases, inflammatory cells may persist within the alveolar septa, but more often the septa resemble those of a normal lung, without inflammatory infiltrate or they are thickened from an interalveolar fibrotic process.

At 29 days of age rat lungs are not quite fully developed. The distal portions of the lung are often not completely alveolarized, demonstrating elastin caps on budding alveolar septa, characteristic of ongoing alveolar septation. We observed many examples of incompletely developed alveolar septa in the TGF- β 1-treated lung, identified under high magnification by the presence of elastin caps (Figure 8). The underdeveloped alveoli and localized fibrotic lesions that developed after TGF- β 1 exposure are both hallmarks of BPD in human newborns and they were not seen after treating pups with the control vector.

Discussion

Branching morphogenesis occurs during early stages of lung development when the respiratory epithelium invades surrounding mesodermal mesenchyme to form the primitive bronchial tree. Bronchial outgrowths continue to branch and develop throughout gestation, eventually

forming the complete bronchial network of the lung, processes that are controlled by finely balanced positive and negative factors.^{12,16}

In the human, at birth, nonrespiratory airways are well developed, however, the alveolar structure of the lung is not yet mature. Extensive alveolarization greatly increases the surface area available for gas exchange by dividing and subdividing the large, premature peripheral air spaces already formed. During this period of growth, the infant lung is particularly sensitive to perturbations in developmental signals. BPD is a common neonatal lung disease that may be caused by such perturbations and is particularly prevalent in premature, very low birth weight newborns. Infants suffering from BPD exhibit a dramatically reduced lung volume,¹⁷ lower levels of alveolarization,¹⁸ and much larger alveolar spaces¹⁹ compared to normal infants.⁶

Chronic inflammation and abnormal tissue repair can lead to pulmonary fibrosis in mature lungs, and similarly, BPD in infants may be caused by inflammation, illustrated by the positive correlation between the occurrence of BPD and high levels of the inflammatory cytokines tumor necrosis factor- α , interleukin (IL)-1 β , IL-6, and IL-8 in amniotic fluid.²⁰ Similar inflammatory mediators are also found in premature neonatal lamb lungs that have been ventilated.²¹ Tracheal aspirates from human premature

Figure 4. Twenty-nine-day-old neonatal rat lung (28 days after treatment). Fibrotic response to AdTGF β 1^{223/225} vector. **A** and **B** were stained with EvG and **C** was stained with Picrosirius Red. Original magnifications: $\times 100$ (**A**, **C**); $\times 200$ (**B**).

infants that have an increased risk of developing BPD contain proinflammatory cytokines such as IL-8, while lacking anti-inflammatory cytokines such as IL-10.²² Increased levels of bioactive TGF- β have also been detected and this can predict the need for prolonged oxygen therapy in extremely premature human infants.²³

Figure 5. Hydroxyproline content of lungs of rats untreated or after intranasal administration of 1×10^8 PFU AdDL70 or AdTGF β 1^{223/225}. Hydroxyproline content was analyzed 21 days (white bars) and 28 days (black bars) after treatment when the pups were 22 and 29 days old, respectively. TGF- β 1 vector-treated group had significantly more hydroxyproline than the control vector group after 28 days, expressed as μ g hydroxyproline per mg of wet lung tissue ($P = 0.078$, one-way analysis of variance; #, $P < 0.05$, *t*-test for TGF- β 1 versus DL70) and was significantly different from day 21 TGF- β 1-treated group (*, $P < 0.05$, *t*-test; $n = 3$ to 5 per group).

Although high O₂ levels may directly contribute to BPD, it has been shown that overinflating the lung during ventilation can also lead to the production of profibrotic cytokines such as tumor necrosis factor- α , basic fibroblast growth factor, and TGF- β 1 as well as extracellular matrix components.^{21,24,25} One might suggest that BPD possibly begins with an inflammatory insult, much like pulmonary fibrosis in adults, followed by the overexpression of TGF- β 1 and deposition of excess extracellular matrix components. The early inflammation could be initiated either by hyperventilation and/or overdistension of the lung, by increased levels of O₂, as well as by exposure of the lung to inflammatory mediators within the amniotic fluid, which may be present because of invading microbes within the mother.^{18,20,24}

To investigate the theory that overexpression of TGF- β 1 plays a key role in the pathogenesis of BPD, we administered neonatal rats intranasally (a process that delivers material to the lung epithelium) with Ad5E1TGF β 1^{223/225}, an adenovirus vector expressing the active form of TGF- β 1, and compared lung histology and collagen content with those seen in controls, either administered a control adenovirus vector or naïve neonatal lungs. Transfer of cytokine and growth factor genes to the lung by adenovirus vectors results in expression of the transgene from infected epithelial cells and lasts for 5 to 10 days, after which the gene is effectively no longer expressed from the vector. Tissue changes seen subsequent to this period are a result of this transient overexpression of transgene.^{9,26–28} The data shown here indicate that transient overexpression of TGF- β 1 induces pathology that closely mimics that seen in the human disease BPD and provides us with an excellent model to study the disease course.

In a study of 46 lung autopsy specimens from infants that suffered negative effects of respiratory distress syndrome, Erickson and colleagues⁵ defined two distinct

Figure 6. Twenty-nine-day-old neonatal rat lung (28 days after treatment). **A, C:** Treatment with AdTGF β 1^{223/225}. **B, D:** Untreated normal rat lung. **A and B** were stained with H&E and **C and D** were stained for α -SMA content. Original magnifications: $\times 100$ (**A, B**); $\times 200$ (**C, D**).

pathological features of BPD and assigned patients into one of three distinct groups. Patients in group 1 have interstitial fibrosis, with extensive scar formation, and a marked inflammatory infiltrate. Group 1 BPD occurs in the very young infant (less than 2 months of age), and is often called early BPD. At the other end of the spectrum, seen in older infants (up to 9 months after birth) are group 3 patients. The lungs of these infants do not show evidence of interstitial fibrosis, but their lungs have reduced numbers of alveolar spaces, resulting in fewer but much larger terminal air spaces. Group 2 BPD has features from both group 1 and group 3, with distal areas of the lung experiencing both pathologies. The progression from interstitial fibrosis to large, underdeveloped airspaces with relatively normal septa is believed to be the

hallmark of the BPD disease process.⁵ The pathology seen in our current studies of neonatal rats closely resembles the photomicrographs seen in the study by Erickson and colleagues⁵ with evidence of areas of fibrosis adjacent to areas of large alveoli (Figures 6 and 7). Consistent with the alveolar hypoplasia seen in the new BPD in low birth weight infants, TGF- β 1-treated animals exhibited numerous areas of primarily large alveoli with an average alveolar size up to twice that of untreated or control vector-treated mice.

Normal rat lungs examined 2 to 4 days after birth demonstrate an incompletely developed lung with a hypercellular interstitium surrounding the airspaces (mean chord length $36.3 \pm 6.0 \mu\text{m}$). Evaluation of high-power fields indicates the cells in the interstitium are primarily noninflammatory cells (data not shown). Rats of an equivalent age that were administered either control adenovirus vector or Ad5E1TGF β 1^{223/225} have similar alveolar spaces at this age (mean chord length 41.7 ± 9.0 and 39.9 ± 9.5 , respectively), but the hypercellular interstitium contains evidence of inflammatory cell infiltration. The lungs of rats treated with either vector were indistinguishable at 3 days after injection (4 days old), and the reaction at this stage is primarily in response to the virus infection (data not shown). After 7 days (8 days old), the

Table 1. Mean Alveolar Chord Length

	Normal (μm)	DL70 treated (μm)	TGF- β 1 treated (μm)
2D	36.3 ± 6.0	41.7 ± 9.0	39.9 ± 9.5
21D	43.4 ± 5.2	43.2 ± 6.9	$63.8 \pm 10.3^*$
28D	31.5 ± 9.5	32.6 ± 8.3	$58.1 \pm 9.1^*$

Values (μm) are mean $\pm$ SD, $n = 10$.

* $P < 0.0001$ versus normal or control vector.

Figure 7. Neonatal rat lungs develop spatially distinct pathologies of fibrosis and inhibited alveolarization 28 days after treatment with AdTGF β 1^{223/225}. **A** was stained with H&E and shows the dense cellular infiltrate within the fibrotic area at the top. **B** is the same section stained with EvG (matrix stains dark purple) and shows the fibrotic area corresponds with the cellular infiltrate, yet is not associated with the underdeveloped alveoli. **C** was stained for α -SMA content. Original magnifications, $\times 50$.

lungs treated with Ad5E1TGF β 1^{223/225} show evidence of new matrix deposition and appear occluded, however, at this time lung hydroxyproline content is not significantly different from control animals. At this stage, these lungs

Figure 8. Neonatal rat lungs 28 days after treatment with AdTGF β 1^{223/225}. Alveolar tissue have elastin caps (dark stain), characteristic of developing alveoli. Stained with EvG. Original magnifications, $\times 400$.

resemble the lungs of infants with early BPD. In rats, the normal initial burst of alveolar formation occurs from days 3 to 8 postnatally.²⁹ Normal untreated lungs at day 8, while still showing a hypercellular interstitium within the alveolar septa, have been undergoing septal thinning and alveolarization and their alveolar spaces have successfully divided to a much greater extent. Administration of active TGF- β 1 to the lungs during this period is likely to interfere with new alveolar formation and as the septa thin, the alveolar space may enlarge. The fact that the mean chord length of day 21 TGF- β 1-treated mice are already markedly enlarged ($63.8 \pm 10.3 \mu\text{m}$) indicates that the enhanced presence of TGF- β 1 may inhibit physiological alveolarization in a similar manner to the inhibition of branching morphogenesis seen in the embryo.^{11,12}

At 29 days of age, normal rat lung approaches maturity with the alveolar septa thinned to involve only a few cells and alveolar development has nearly reached adult levels. Rats injected with control virus had also recovered, and have apparently normally developed lungs at this time, although some lung specimens did exhibit interstitial infiltrates, which have probably persisted since vector administration. It would seem likely that the relatively low-level inflammatory insult caused by the adenovirus vector itself is enough to cause this limited pathology.³⁰ Nonetheless, the hydroxyproline content is nearly identical between the two control groups and there is no histological evidence of fibrosis and the alveolar space size is similar in both groups. Whether the phagocytic influx is necessary for the development of fibrosis subsequent to the expression of active TGF- β 1 cannot be ascertained from these data, however, having expressed more than 20 cytokines in this manner in the lung, only two, IL-1 β and TGF- β 1, exhibit the ability to initiate progressive and widespread fibrosis.^{9,31}

In contrast, neonatal rats at 28 days after administration of Ad5E1TGF β 1^{223/225} (29 days of age) showed pathology closely resembling either mid-stage or late-stage human BPD. Areas with interstitial fibrosis demonstrate marked matrix deposition, leading to significantly higher

hydroxyproline content of the treated lungs ($\sim 150\%$ of control levels). Stimulation of matrix production by TGF- β includes a number of matrix glycoproteins such as fibronectin, recently shown to have a direct correlation with the extent of fibroproliferation in BPD.³² Often adjacent to fibrotic areas are large, alveolar spaces surrounded by normal-appearing septa with only limited cellularity. Although the septa have apparently undergone normal thinning, there does not appear to have been continued alveolar division, resulting in the enlarged alveolar spaces seen, characteristic of a more advanced stage of disease progression. The marked difference in mean alveolar space size seen between the TGF- β 1-treated animals and either control group (2 times higher) confirms the morphological appearances seen in Figure 4. The appearance of α -SMA-positive myofibroblasts within the interstitium of enlarged alveoli might indicate a physiological impact of these contractile cells on alveolar function (Figure 6). In addition, within the fibrotic areas, α -SMA-positive cells would be major contributors to the deposition of matrix. Thus these cells may play different roles in different tissue sites within the lung.

At 29 days of age, while approaching lung maturity, untreated normal rats show evidence of continuing alveolar division and differentiation, as protruding alveolar septations end in elastin caps, which is a feature characteristic of budding septa. These sites seem to be equally prevalent in all groups, and show that the alveolar content of rats are not yet fully developed to adult levels at this time. The heterogeneity of the response in terms of the disease course would likely evolve throughout a longer time frame. It will be interesting to observe the final stage of the disease course at a later time point, and whether the hydroxyproline levels decrease in accordance with the resolution of interstitial fibrosis in this model, as predicted for human disease by Erickson and colleagues.⁵

Our results suggest that TGF- β 1 may continue to act as a negative regulator of normal alveolar differentiation in the newborn. We have shown previously in embryonic lungs that overexpression of active TGF- β 1 acts on embryonic bronchial epithelium to prevent branching morphogenesis,¹⁰ possibly by negatively regulating positive signaling through other growth factor receptors.³³ Similarly, fetal stage transgenic epithelial expression of TGF- β 1 arrests lung epithelial and vascular morphogenesis in the pseudoglandular stage.³⁴ Recently, a regulated transgenic mouse model of neonatal active TGF- β 1 expression from lung epithelium was presented that showed similar features consistent with BPD.³⁵

The adenovirus vector delivery of constitutively active TGF- β 1 to neonatal rat lungs initiates a disease course that closely resembles BPD in human newborns. This neonatal rat model of BPD is characterized by both alveolar hypoplasia and interstitial fibrosis. Thus, the model has features of both classical and new forms of BPD. The development of an efficient intranasal delivery method makes this a promising model to further evaluate the role of other growth factors and cytokines in normal and altered neonatal lung development. Ideally, this model will be useful in the development of novel therapeutic strat-

egies that might ameliorate or prevent the negative effects of BPD on the human neonatal lung.

Acknowledgments

We thank Duncan Chong, Xueya Feng, and Mary Jo Smith for careful technical assistance and expertise.

References

1. Bancalari E: Changes in the pathogenesis and prevention of chronic lung disease of prematurity. *Am J Perinatol* 2001, 18:1–9
2. Jobe AH, Ikegami M: Prevention of bronchopulmonary dysplasia. *Curr Opin Pediatr* 2001, 13:124–129
3. Northway WH, Rosan RC, Porter DY: Pulmonary disease following respiratory therapy of hyaline membrane disease. Bronchopulmonary dysplasia. *N Engl J Med* 1967, 276:357–368
4. Jobe AH: The new BPD: an arrest of lung development. *Pediatr Res* 1999, 46:641–643
5. Erickson AM, de la Monte SM, Moore GW, Hutchins GM: The progression of morphologic changes in bronchopulmonary dysplasia. *Am J Pathol* 1987, 127:474–484
6. Jobe AH, Bancalari E: Bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 2001, 163:1723–1729
7. Selman M, King Jr TE, Pardo A: Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann Intern Med* 2001, 134:136–151
8. Gauldie J, Kolb M, Sime PJ: A new direction in the pathogenesis of idiopathic pulmonary fibrosis? *Respir Res* 2002, 3:1–3
9. Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J: Adenovector-mediated gene transfer of active TGF- β 1 induces prolonged severe fibrosis in rat lung. *J Clin Invest* 1997, 100:768–776
10. Zhao J, Sime PJ, Bringas Jr P, Gauldie J, Warburton D: Epithelium-specific adenoviral transfer of a dominant-negative mutant TGF β type II receptor stimulates embryonic lung branching morphogenesis in culture and potentiates EGF and PDGF-AA. *Mech Dev* 1998, 72:89–100
11. Zhao J, Sime PJ, Bringas P, Tefft JD, Buckley S, Bu D, Gauldie J, Warburton D: Spatial-specific TGF- β 1 adenoviral expression determines morphogenetic phenotypes in embryonic mouse lung. *Eur J Cell Biol* 1999, 78:715–725
12. Warburton D, Schwarz M, Tefft D, Flores-Delgado G, Anderson KD, Cardoso WV: The molecular basis of lung morphogenesis. *Mech Dev* 2001, 92:55–81
13. Van Ginkel FW, Liu C, Simecka JW, Dong JY, Greenway T, Frizzell RA, Kiyono H, McGhee JR, Pascual DW: Intratracheal gene delivery with adenoviral vector induces elevated systemic IgG and mucosal IgA antibodies to adenovirus and beta-galactosidase. *Hum Gene Therapy* 1995, 6:895–903
14. Woessner JF: The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys* 1961, 93:440–447
15. Zheng T, Zhu Z, Wang Z, Homer RJ, Ma B, Riese Jr RJ, Chapman HA, Shapiro SD, Elias JA: Inducible targeting of IL-13 to the adult lung causes matrix metalloproteinase- and cathepsin-dependent emphysema. *J Clin Invest* 2000, 106:1081–1093
16. Hilfer S: Morphogenesis of the lung: control of embryonic and fetal branching. *Annu Rev Physiol* 1996, 58:93–113
17. Jackson JC, Standaert TA, Truog WE, Murphy JH, Palmer S, Chi EY, Woodrum DE, Watchko JF, Hodson WA: Changes in lung volume and deflation stability in hyaline membrane disease. *J Appl Physiol* 1985, 59:1783–1789
18. Chambers HN, van Velzen D: Ventilator-related pathology in the extremely immature lung. *Pathology* 1989, 21:79–83
19. Jobe A, Ikegami M: Mechanisms initiating lung injury in the preterm. *Early Hum Dev* 1998, 53:81–94
20. Yoon BH, Romero R, Jun JK, Park KH, Park JD, Ghezzi F, Kim BI: Amniotic fluid cytokines (interleukin-6, tumor necrosis factor- α , interleukin-1 β , and interleukin-8) and the risk for the development of

- bronchopulmonary dysplasia. *Am J Obstet Gynecol* 1997, 177:825–830
21. Naik AS, Kallapur SG, Bachurski CJ, Jobe AH, Michna J, Kramer BW, Ikegami M: Effects of ventilation with different positive end-expiratory pressures on cytokine expression in the preterm lamb lung. *Am J Respir Crit Care Med* 2001, 164:494–498
22. Jones CA, Cayabyab RG, Kwong KY, Stotts C, Wong B, Hamdan H, Minoo P, de Lemos RA: Undetectable interleukin (IL)-10 and persistent IL-8 expression early in hyaline membrane disease: a possible developmental basis for the predisposition to chronic lung inflammation in preterm newborns. *Pediatr Res* 1996, 39:966–975
23. LeCarr C, Cayabyab R, Buckley S, Morrison J, Kwong KY, Warburton D, Ramanathan R, Jones CA, Minoo P: Bioactive transforming growth factor-beta in the lungs of extremely low birthweight neonates predicts the need for home oxygen supplementation. *Biol Neonate* 2000, 77:217–223
24. Garland JS, Buck RK, Allred EN, Leviton A: Hypocarbia before surfactant therapy appears to increase bronchopulmonary dysplasia risk in infants with respiratory distress syndrome. *Arch Pediatr Adolesc Med* 1995, 149:617–622
25. Berg JT, Fu Z, Breen EC, Tran HC, Mathieu-Costello O, West JB: High lung inflation increases mRNA levels of ECM components and growth factors in lung parenchyma. *J Appl Physiol* 1997, 83:120–128
26. Xing Z, Ohkawara Y, Jordana M, Graham FL, Gauldie J: Transfer of granulocyte-macrophage colony-stimulating factor gene to rat lung induces eosinophilia, monocytosis, and fibrotic reactions. *J Clin Invest* 1996, 97:1102–1110
27. Xing Z, Tremblay GM, Sime PJ, Gauldie J: Overexpression of granulocyte-macrophage colony stimulating factor induces pulmonary granulation tissue formation and fibrosis by induction of transforming growth factor- β 1 and myofibroblasts accumulation. *Am J Pathol* 1997, 150:59–66
28. Sime PJ, Marr RA, Gauldie D, Xing Z, Hewlett BR, Graham FL, Gauldie J: Transfer of TNF α to rat lung induces severe pulmonary inflammation and patchy interstitial fibrogenesis with induction of TGF- β 1 and myofibroblasts. *Am J Pathol* 1998, 153:825–832
29. Meyrick B, Reid L: Pulmonary arterial and alveolar development in normal postnatal rat lung. *Am Rev Respir Dis* 1982, 125:468–473
30. Zsengeller ZK, Ross GF, Trapnell BC, Szabo C, Whitsett JA: Adenovirus infection increases iNOS and peroxynitrite production in the lung. *Am J Physiol* 2001, 280:L503–L511
31. Kolb M, Margetts PJ, Anthony DC, Pitossi F, Gauldie J: Transient expression of IL-1 β induces acute lung injury and chronic repair leading to pulmonary fibrosis. *J Clin Invest* 2001, 107:1529–1536
32. Sinkin RA, Roberts M, LoMonaco MB, Sanders RJ, Metlay LA: Fibronectin expression in bronchopulmonary dysplasia. *Pediatr Dev Pathol* 1998, 1:494–502
33. Miettinen PJ, Warburton D, Bu D, Zhao JS, Berger JE, Minoo P, Koivisto T, Allen L, Dobbs L, Werb Z, Derynck R: Impaired lung branching morphogenesis in the absence of functional EGF receptor. *Dev Biol* 1997, 186:224–236
34. Zeng X, Gray M, Stahlman MT, Whitsett JA: TGF-beta1 perturbs vascular development and inhibits epithelial differentiation in fetal lung in vivo. *Dev Dyn* 2001, 221:289–301
35. Vincencio AG, Lee CG, Eickelberg O, Haddad GG, Elias J: Pathologic characteristics of TGF β 1 overexpression in neonatal mouse lung. *Am J Respir Crit Care Med* 2003, 167:A594 (Abstract)