Susceptibility to Asbestos-Induced and Transforming Growth Factor-β_1–Induced Fibroproliferative Lung Disease in Two Strains of Mice

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Pulmonary fibrosis (PF) is caused by a number of inhaled agents, as well as by some drugs and toxic particles. The elaboration of certain peptide growth factors is thought to be key to the development of this disease process. In addition, genetic susceptibility plays a role in the development of PF. For instance, we have previously shown that the 129J strain of mice is resistant, whereas the C57BL/6 strain is highly susceptible, to asbestos-induced fibrosis. To pursue this further, in one mouse model, we crossed the 129J strain to the C57BL/6 strain to produce an F1 generation and subsequently backcrossed the F1 mice to the inbred founders. This backcross to the 129 inbred strain produced ∽ 25% of the offspring with a phenotype that was protected from the fibrogenic effects of inhaled asbestos fibers. In the second model, both strains of mice were treated intratracheally with an adenovirus vector (AdV), which transduces expression of active transforming growth factor (TGF)-β_1 in the lungs, producing fibroproliferative lung disease. Compared with C57 mice, a significant number of 129 strain mice exhibited at least a 1-wk delay in the fibroproliferative response to TGF-β_1 expression at three concentrations of virus. These findings suggest that certain sequences in a gene or a cluster of genes in the 129 mouse strain impart a phenotype in which there is a delay in, or protection from, the development of lung fibrogenesis.

Various mouse strains are commonly used to provide models of human lung disease. Among these are interstitial lung disease induced by inhaled particles (1, 2) or by viral vectors that transduce expression of peptide growth factors (3, 4). These studies are performed in attempts to understand the molecular mechanisms that mediate the development of idiopathic pulmonary fibrosis (IPF). This disease process has multiple etiologies and afflicts millions of individuals worldwide (5). It has been difficult to sort out the mechanisms of disease because so many mediators are elaborated as the process progresses. The animal models are invaluable in this regard and point to a group of peptide growth factors that may be largely responsible for the development of IPF (4, 6). For example, asbestos exposure induces rapid expression of platelet-derived growth factor (PDGF) isoforms (7), tumor necrosis factor (TNF)-α (8), transforming growth factor (TGF)-β (9), and TGF-α (10). These are all overexpressed at sites of lung injury but not by normal cells. In addition, in a model of bleomycin-induced IPF, an antibody to TGF-β blocked some disease development (11). Mice that have had both receptors for TNF-α knocked out by heterologous recombination fail to develop pulmonary fibrosis consequent to treatment with asbestos (8), silica, or bleomycin (12). As other examples, animals treated with the peptides intratracheally (13, 14) or by an adenovirus vector, as mentioned above (3, 4, 15), develop pulmonary fibrosis, demonstrating further the roles of these peptide growth factors in mediating the process.

Pursuant to this "growth factor hypothesis," we are using selected mouse strains in attempting to understand the pathogenesis of IPF as well as the genetic basis for "susceptibility" in a given animal's response to a fibrogenic agent. Thus, in any population of mice exposed to fibrogenic agents such as asbestos or TGF-β, there is a range in the degree of response, just as has been observed in populations of occupationally exposed workers (16). To sort out the basis of this susceptibility is enormously important from a public health standpoint, and it will be a difficult task because of the many thousands of genes that could be involved. We are attempting to take advantage of observations made in a strain of inbred mice known as the 129J. This strain has been used for decades in skin-painting experiments (17) and as a source of embryonic stem cells (18). In the skin painting experiments, the 129 mice exhibited a reduced dermal scarring response. In our laboratory, we showed that these mice develop a reduced fibroproliferative response to inhaled asbestos, accompanied by reduced expression of TGF-β_1 at sites of fiber deposition (19); and primary lung fibroblasts from the 129J mice maintained in culture proliferate more slowly, make less collagen, and respond less to PDGF, TGF-β, and TNF-α (20). We are asking if these mice could help us understand the genetic basis for an apparent "protected" phenotype.

In our asbestos model, the fibrogenic lesions are predictable and quantifiable (1). We know that the asbestos-induced lesions in the 129 mice are reduced compared with C57 mice, and the difference persists through 1 mo after exposure (19). Thus, in the set of experiments presented

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Abbreviations: adenovirus, AdV; bronchoalveolar lavage, BAL; Bromodeoxyuridine, BrdU; enzyme-linked immunosorbent assay, ELISA; hematoxylin and eosin, H&E; idiopathic pulmonary fibrosis, IPF; phosphate-buffered saline, PBS; platelet-derived growth factor, PDGF; pulmonary fibrosis, PF; plaque-forming units, pfu; transforming growth factor, TGF; tumor necrosis factor, TNF.

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here, we crossed 129 and C57 mice to produce an F1 generation that was then backcrossed to either the 129 or C57 founders. If specific sequence changes in a gene or cluster of genes are controlling this protection from asbestos, it should separate out into a predicted number of offspring that results from the F1 × 129 backcross. We show here that 26% of these asbestos-exposed offspring failed to develop asbestos-induced lung lesions, whereas only one out of 21 (4.7%) mice from the F1 × C57 cross had no disease.

In addition, inasmuch as TGF-β (as discussed above) is likely to play a major role in the development of IPF regardless of the inducing agent, we asked if differences in disease development could be identified in the two mouse strains consequent to transduction of TGF-β overexpression by an adenovirus vector. We show here that, again, this time even with direct application of the growth factor to the lung parenchyma, there is a reduced degree of disease and a clear delay in the development of fibrogenesis in the 129 mouse strain early in the disease process at three concentrations of virus.

Materials and Methods

Asbestos Exposure

Eight- to twelve-week-old C57BL/6 or 129 mice (Jackson Laboratories, Bar Harbor, ME) were exposed to an aerosol of chrysotile asbestos as described earlier (19). The asbestos exposures were performed in two separate experiments. In one experiment, the C57BL/6, 129, or F1 (C57BL/6 × 129) mice received a single exposure of 16.3 mg/m^3 asbestos for 5 h. In a second experiment the (F1 × 129), (F1 x C57BL/6), or C57BL/6 mice were exposed to asbestos for 5 h each day for two consecutive days (fiber concentrations, 12.8 and 17.5 mg/m^3, respectively). The number of animals per group in the intercross and backcross groups ranged from 19–40. Groups of 3–5 unexposed mice (all strains and crosses) were maintained in room air as controls. The animals were killed 48 h after exposure by an intraperitoneal injection of 0.9 ml/kg of body weight of Ketaset followed by exsanguination through the renal artery. Lung fixation and histologic evaluation were performed as described below.

Recombinant Adenovirus Vectors: Propagation and Purification

Replication-deficient, human adenovirus type-5 genome-based recombinant virus expressing the biologically active porcine TGF-β_1 (AdV TGF-$\beta_1^{223/225}$) was provided by Dr. Jack Gauldie (McMaster University, Hamilton, ON, Canada). Replication-deficient, human adenovirus type-5 genome-based recombinant viruses carrying either an unrelated DNA sequence in place of the coding region for TGF-β_1 (AdVMG3) was constructed in the laboratory of Dr. David Curiel (University of Alabama at Birmingham, Birmingham, AL) and kindly provided by Dr. Deborah Sullivan (Tulane University Health Sciences Center, New Orleans, LA).

The methods for propagation and purification of the recombinant adenoviruses were as previously described (21, 22). The viruses were purified by two rounds of CsCl–gradient centrifugation and the CsCl was removed by chromatography of the virus suspension using Econo-Pac 10 DG desalting columns (Bio-Rad Laboratories, Hercules, CA). Fractions of virus in 10% glycerol in phosphate-buffered saline (PBS) were pooled. Total particles of virus were measured by a spectrophotometric value at 260 nm and infectious particles were assessed by measuring plaque-forming units (pfu) using the method described (21) except 911 cells (23) were used instead of 293 cells and plaques were counted on Days 4–5.

Viral Instillation

Six- to eight-week-old male, pathogen-free 129 or C57BL/6 mice weighing 20–25 g were purchased from Jackson Laboratories. The animals were housed in a temperature- and light-controlled room with free access to food and water. Mice were anaesthetized intraperitoneally with 0.8 ml/kg of body weight of a solution of Ketaset (90.9 mg/ml ketamine and 9.1 mg/ml xylazine; Fort Dodge Laboratories Inc, Fort Dodge, IA) before making a midline incision of ∽ 1 cm in the neck and visualizing the trachea by carefully moving the musculature. Known concentrations of the virus in 50 μl of sterile PBS was instilled intratracheally using a 50-μl Hamilton syringe (Hamilton Co., Reno, NV) attached to a 33-gauge needle. The incision was closed with wound clips (Clay Adams; Becton-Dickinson, Sparks, MD) and the animals were monitored throughout the course of the experiment.

Lung Fixation and Histologic Evaluation

Anaesthetized mice (n = 3–5 per group) were instilled with 5 × 10^7, 10^8, or 10^9 pfu of AdTGF$\beta_1^{223/225}$ (AdVTGFβ_1) or 5 × 10^7 or 10^8 pfu of AdVMG3 (AV) in 50 μl intratracheally as described above. At 4, 7, 14, and 28 d after treatment, the animals were killed by intraperitoneal injection of 0.9 ml/kg of body weight of Ketaset, followed by exsanguination through the renal artery. After exposing the chest cavity, the right main bronchus was sutured at the base of the main stem and the right lung was clipped off, snap-frozen in liquid nitrogen, and stored at –70°C for mRNA analysis. The left lung was perfused with 10% neutral buffered formalin (Sigma Chemical Co., St. Louis, MO) at a pressure of 25 cm H_2O for 15–20 min, removed from the animal, and placed in fresh 10% neutral buffered formalin for 16–20 h at 4°C before processing and embedding. Sections (4 μm) from each sample were stained with Hematoxylin and Eosin (H&E) for histopathologic evaluation.

The same procedure was followed for histopathologic evaluation of lung tissue from mice exposed to asbestos and killed 48 h after exposure.

Severity of disease pathology was quantified by microscopic evaluation of each H&E section in a blinded method as described previously (7–10, 19). Two sections were examined from each animal and the severity scores assigned were as follows: 0 (normal); 1 (slight peribronchiolar and/or perivascular inflammation); 2 (spread of inflammation to parenchyma); 3 (widespread inflammation and fibrogenesis); 4 (severe diffuse inflammation and fibrogenesis).

Collection of Bronchoalveolar Lavage

Anaesthetized mice (n = 3–5 per group) were instilled with 5 × 10^7 or 10^8 pfu of AdTGF$\beta_1^{223/225}$ (AdVTGFβ_1) or AdVMG3 (AV) in 50 μl of sterile PBS as described above. At 4, 7, 14, and 28 d after treatment, bronchoalveolar lavage (BAL) samples were collected by instillation of five 0.8-ml aliquots of cold lavage buffer (PBS + 0.4 mM EDTA), one aliquot at a time, using a 1-ml syringe attached to a 20-gauge intravenous catheter (Johnson and Johnson Medical, Arlington, TX) inserted into the trachea. The first aliquot of lavage buffer recovered was kept separate and the rest were pooled. All volumes of recovered lavage buffer were recorded.

Cytologic Evaluation of Inflammatory Cell Accumulation in the Lung

All BAL samples collected were centrifuged at 4,000 rpm for 5 min. at 4°C to separate the cells from the supernatant. The supernatant from the first lavage was stored at –70°C in 0.2-ml aliquots for TGF-β_1 protein analysis (*see below*). The cells from all five aliquots of BAL fluid were pooled by resuspension in 0.4 ml of lavage buffer, and total cell numbers in the BAL were determined using a hematocytometer. Cells (5×10^4 from each sample) were transferred onto glass slides using a Cytospin centrifuge (Shandon, Pittsburgh, PA). Cell smears thus prepared were dried briefly and stained with Diff Quick (Baxter, McGaw Park, IL) for differential cell staining. Differential cell counts were made by counting 500 cells/prep in random fields on a light microscope at ×400 magnification.

Quantitation of Active and Latent TGF-β_1 Expression in the Lung

TGF-β_1 protein in the lung after instillation of AV or AdVTGFβ_1 was measured by enzyme-linked immunosorbent assay (ELISA) of the BAL fluid supernatant using a commercially available kit (Promega Corporation, Madison, WI) according to the manufacturer's instructions. The assay measures only active TGF-β_1, but acidification of the BAL supernatant activates the latent TGF-β_1, thus allowing a measurement of total TGF-β_1 and calculation of the latent TGF-β_1 content.

Detection and Quantitation of DNA synthesis: Bromodeoxyuridine Labeling

Four to five hours before killing and lung tissue fixation for paraffin embedding, all mice were injected intraperitoneally with a solution of 5′-Bromodeoxyuridine (BrdU), (Sigma Chemical Co) pH 7.4, at a concentration of 40–50 mg/kg of body weight (24) in a volume of 0.5 ml sterile PBS. Immunohistochemistry was performed on deparaffinized lung tissue sections as previously described (8) using a rat monoclonal antibody against BrdU (Harlan Sera laboratories Ltd., Loughbrough, UK) and examined by light microscopy.

Quantitation of BrdU labeled sections were performed as follows: positively stained cells from defined anatomic locations of the lung were counted (24, 25) by light microscopy at ×400 magnification. Three to five fields were counted for each location. Defined locations were as follows:

1. Epithelial cells: (*i*) airway epithelial (AE) cells in the terminal bronchioles (positive cells in the first 100 epithelial cells lining the bronchiole, starting at the alveolar duct); (*ii*) cross-sectional airway epithelial cells (CAE, positive and total number of epithelial cells).
2. Interstitial cells: (*i*) airway interstitial (AI) cells in the terminal bronchioles (positive and total number of interstitial cells beneath the first 100 epithelial cells counted in 1(*i*); (*ii*) cross-sectional airway interstitial (CAI) cells (positive and total number of interstitial cells beneath the first 100 epithelial cells counted in 1(*ii*).
3. Parenchymal cells: all parenchymal (epithelial and interstitial) cells in a randomly selected area were counted. Three to five fields within the area were chosen by moving the stage by 0.5 μm. (*i*) Disease area (involved parenchyma, IP); (*ii*) normal area (uninvolved parenchyma, UP).
4. Inflammatory cells: cells in peribronchiolar and perivascular inflammatory loci (inflammatory foci, IF) were counted in randomly selected areas. BrdU-positive cell numbers are reported as a percentage of total cells counted for each location.

β-Galactosidase Expression in C57BL/6 and 129 Mouse Strains

To determine whether or not there were differences in degree of expression of a common gene, 5×10^8 pfu of an adenoviral vector carrying the coding region for *Escherichia coli* β-galactosidase (*lacZ*) gene (rAdVCMVLacZ) were instilled intratracheally into C57BL/6 and 129 mice (n = 3–7) as described above. The animals were killed after 7 d of treatment and the lungs were perfused with 5 ml of saline injected through the pulmonary artery to clear the lungs of all blood. The lung tissue was homogenized in 0.5 ml of reporter lysis buffer (Promega) using a tissue homogenizer. The homogenate was centrifuged and the supernatant was used to measure the β-galactosidase activity using a commercial β-galactosidase enzyme assay system (Promega) according to the manufacturer's instructions. A colorimetric value was obtained for enzyme activity by measuring the absorbance at 405 nm using a 96-well plate reader.

Statistical Analysis

All data are reported as means ± SEM. Differences between treatment groups were analyzed by an unpaired t test or an unpaired nonparametric test (software used: Instat for MacIntosh 1,14; Graphpad Software, San Diego, CA). The differences were considered statistically significant when $P < 0.05$.

Results

Asbestos Exposure

These experiments provide the opportunity to quantify a well-defined fibrogenic lesion in the two mouse strains after an F1 generation is backcrossed to either the 129 or C57 strain.

Initial asbestos-induced pulmonary fibrosis develops within 48 h of a single 5-h exposure to the fibers in rats and mice (1, 7) (Figure 1). We quantified the degree of lesion development in populations of the 129 inbred (1.8 ± 0.3, n = 5) and C57 strains (2.7 ± 0.1, n = 10). Unexposed mice rarely (2 out of 18) have a score greater than 1 (Figures 1 and 2). This difference in response between the two inbred strains had been shown previously in separate experiments (19) and is repeated to control for potential differences in exposure conditions. Here we show that crossing the two strains results in an F1 generation that responds to asbestos with a broad range of scores (Figure 2). Whereas the C57 animals all have scores of 2 or more, and the 129 mice typically (4/5) have scores of 2 or less, the F1 offspring ranged from 0–3 (2.18 ± 1.2, n = 31). When F1 mice were backcrossed to the C57 strain, only one out of 21 (4.7%) offspring was classified as having no clear asbestos lesions (2.4 ± 0.1, n = 21) (Figure 2). In comparison, the offspring resulting from the F1 × 129 backcross had 5 of 19 (26.3%) animals with no apparent asbestos-induced lesions (1.6 ± 0.0.6, n = 19) (Figure 2). Statistical analyses (unpaired t test or unpaired nonparametric test) were performed to compare the two backcrossed groups, with and without the two outlying values removed. The differences (using either test) were highly significant, with $P < 0.0009$.

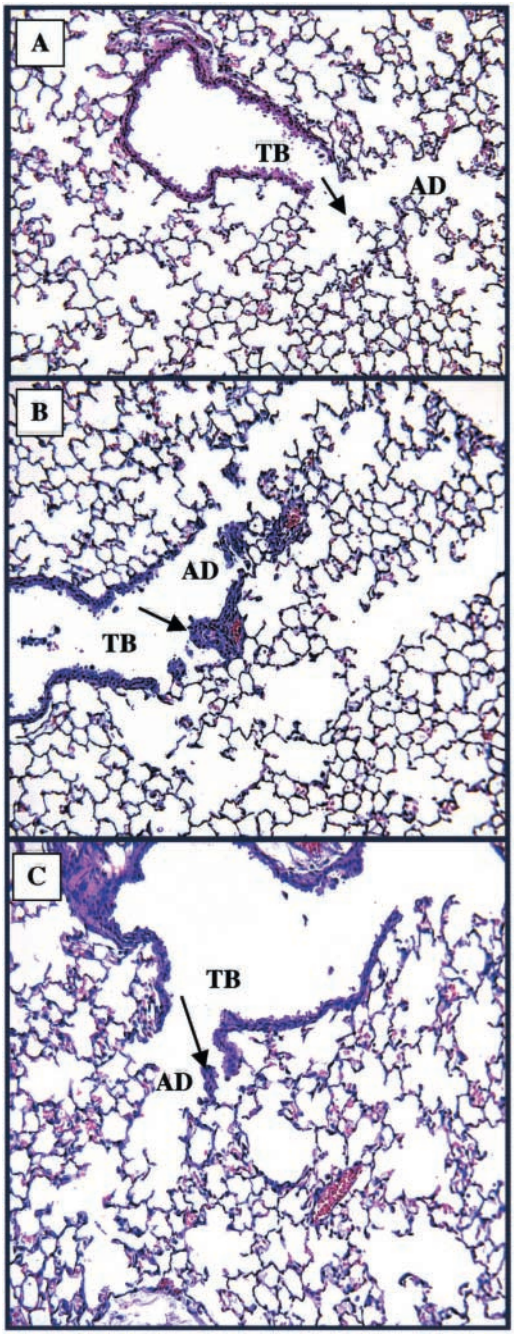

Figure 1. Histopathology of asbestos-exposed lung tissue in C57BL/6 and 129 mice: 48 h after exposure, C57BL/6 or 129 mouse lungs were fixed and sections stained with H&E for assessment of histopathology. Bronchiolar-alveolar duct bifurcations, the sites of fiber deposition, and initial lesion development are marked by an *arrow* (*magnification:* ×400). TB, terminal bronchiole; AD, alveolar duct. (*A*) C57BL/6, air exposed (control; score = 0). (*B*) C57BL/6 (score = 3). (*C*) 129 (score = 1).

TGF-β Overexpression

Degree of fibroproliferation by histopathology scoring. An adenovirus vector transducing expression of biologically-active TGF-β_1 (4) was used to induce fibroproliferation in 129 and C57 mice. Three concentrations of virus (5×10^7, 10^8, and 10^9 pfu) were assessed at 7 d after exposure (Figure 3). The 10^8 and 10^9 doses were evaluated by histopathology at 7 and 14 d (Figures 3 and 4). Degree of disease induced by the 10^8 pfu dose was quantified at 4–28 d after treatment (Figure 5), and the 5×10^7 dose was quantified at Day 7 only. The histopathologic findings were the same, as we reported previously in a study to establish responses to a broad dose range in C57 mice (26). At both the 10^8 and 10^9 pfu doses, the histopathology appeared worse in the C57 than in the 129 mice at 7 d after treatment. Because the 10^9 pfu dose was lethal for most of the 129 mice, quantitation was performed with the 10^8 pfu dose (Figure 5). Diffuse interstitial inflammation and fibrogenesis became obvious in the C57 mice at Day 4 and was clearly present by 7 d after treatment, with diminution of the disease by the 28-d time period (Figure 5). The striking difference in this study is the finding that by 7 d, at 5×10^7 pfu, 8 of the 13 129 mice exhibited no evidence of disease (score of 0), and at 10^8 pfu, 4 of 11 129 mice were essentially normal (score of 1 or less) (Figure 5). At both concentrations of vector, all 25 C57 mice had scores of 2 or greater at 7 d (Figure 5). Interestingly, by Day 14, all but two of the mice (both 129 and C57) exhibited scores greater than 1 (Figure 5). A dose of 10^8 pfu of the control vector alone produced no scores greater than 1 in any of the animals at any time point (data not shown).

BrdU Incorporation

The degree of lung cell proliferation in the two mouse strains was assessed by quantitation of BrdU incorporation (Figure 6). At 7 and 14 d after treatment, the percentages of BrdU-positive cells were just as predicted by the histopathology, with the C57 mice exhibiting significantly more proliferation at every anatomic region studied at 7 d, but by 14 d, the 129 mice appeared to have more disease (Figure 6). The airway epithelium at both time points and in both strains exhibited a reduced proliferative response compared with the interstitium, probably due to the presence of abundant TGF-β_1 as we reported previously in separate studies (15, 26).

Quantitation of TGF-β Protein

Quantitation of active and latent TGF-β_1 in lavaged fluids showed significantly more protein at Day 4 from the lavage of C57 mice than from the 129 animals (Figure 7). At the 7-d time point, there is significantly more TGF-β_1 than in the control mice, but differences between the two strains fail to reach statistical significance (Figure 7).

β-Galactosidase Expression in C57BL/6 and 129 Mouse Strains

To confirm the ability of both strains of mice to express a transgene from an adenoviral vector with equal efficiency, β-galactosidase enzyme expression was used. The absorbance values from a range of samples were used to generate a standard curve, and the enzyme activities of unknown samples were measured in milliunits of β-galactosidase. The mean ± SEM values for C57BL/6 (1.06 ± 0.02, $n = 3$) and 129 (0.86 ± 0.10, $n = 7$) did not differ significantly. The

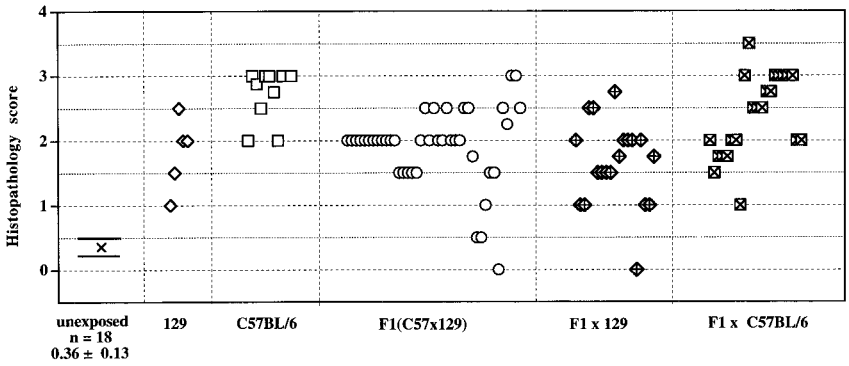

Figure 2. Histopathology scores of C57BL/6 and 129 intercross and F1 backcross hybrids 48 h after exposure to asbestos: Severity of disease was quantified by microscopic evaluation of H&E sections in a blinded method as reported previously in References 7–10 and 19. Severity scores: 0 = normal; 1 = slight peribronchiolar and/or perivascular inflammation; 2 = spread of inflammation to parenchyma; 3 = widespread inflammation and fibrogenesis; 4 = severe diffuse inflammation and fibrogenesis. Compared with C57 BL/6, 129 mice showed significantly lower disease pathology in wild-type ($P = 0.003$), F1 hybrids, and F1x progenitor backcross hybrids ($P = 0.0009$).

statistical significance was analyzed by both an unpaired *t* test and an unpaired nonparametric test.

Cells Recovered by Lavage

The time of peak inflammatory cell accumulation, 7 d for C57BL/6 and 14 d for 129 mice (Figure 8), was reflected by the cells recovered in BAL. When comparing individual cell types, the number of macrophages was consistently higher in 129 mice through Days 4–28, the difference being statistically significant only on Day 14, when the disease was worse in the 129 mice (Figure 8). On Day 7, neutrophils and lymphocytes were the dominating cell types in C57 s (Figure 8), and the numbers were significantly higher compared with 129 mice.

Discussion

We have shown that two fibrogenic agents, asbestos fibers delivered by an aerosol generator and TGF-β_1 transduced by an adenovirus vector, cause interstitial inflammation and

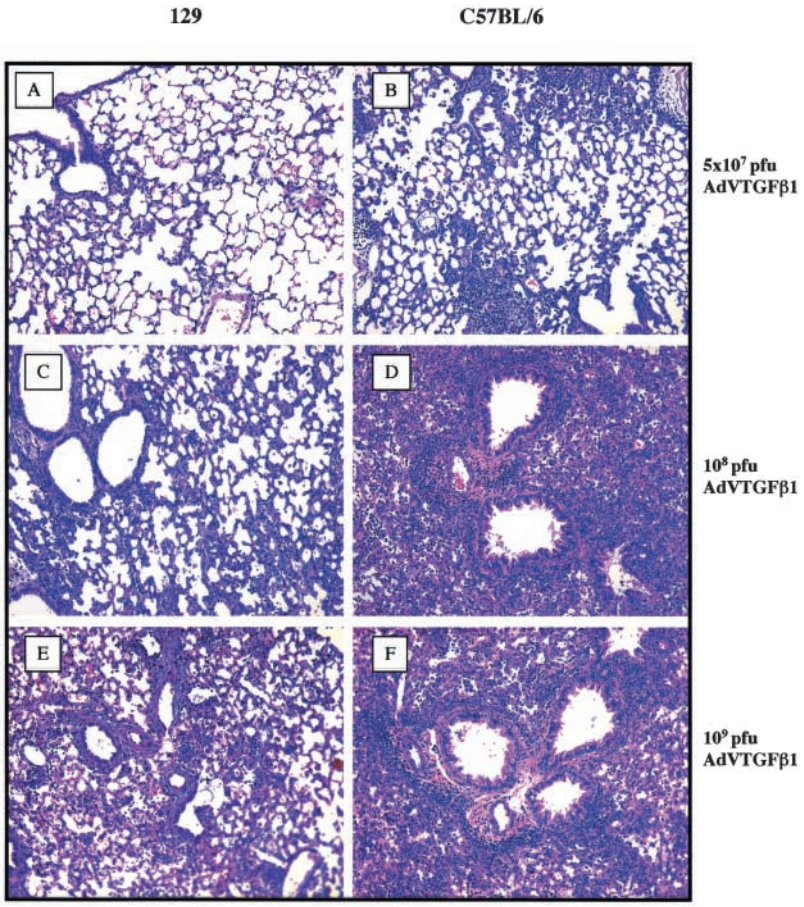

Figure 3. Comparison of histologic evaluation of H&E-stained lung sections of mice that received 5×10^7–10^9 pfu of AdVTGFβ_1. Seven days after tracheal instillation; for three viral doses used (10^9, 10^8, and 5×10^7 pfu) C57BL/6 mice showed an increased disease severity compared with 129 mice (*magnification:* ×400). Lungs of mice receiving the same dose of control virus showed normal histology (*see* Figure 4). (*A* and *B*) 5×10^7 pfu AdVTGFβ_1; (*C* and *D*) 10^8 pfu AdVTGFβ_1; (*E* and *F*) 10^9 pfu AdVTGFβ_1.

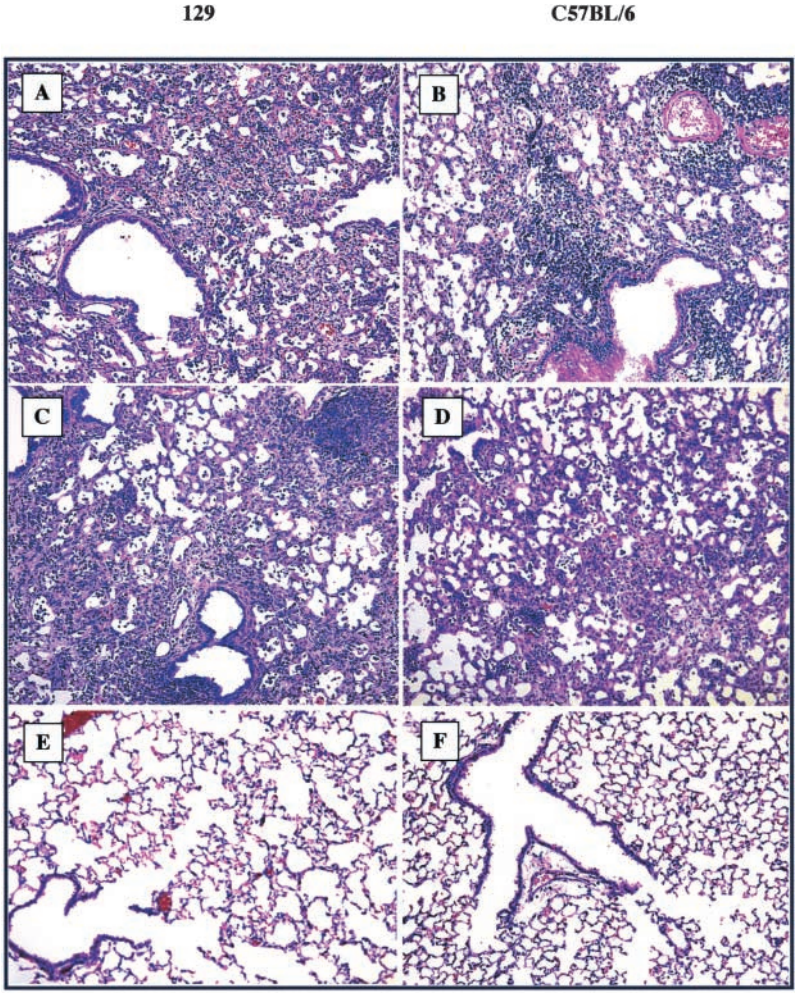

Figure 4. Comparison of histologic evaluation of H&E-stained lung sections of mice that received 10^8 or 10^9 pfu of AdVTGFβ_1. Fourteen days after tracheal instillation; 129 and C57 mice showed a range of disease severity on Day 14. Lungs of mice receiving the same dose of control virus (AV) showed normal histology. (*magnification:* ×400). (*A* and *B*) 10^8 pfu AdVTGFβ_1; (*C* and *D*) 10^9 pfu AdVTGFβ_1; (*E* and *F*) 10^9 pfu AV.

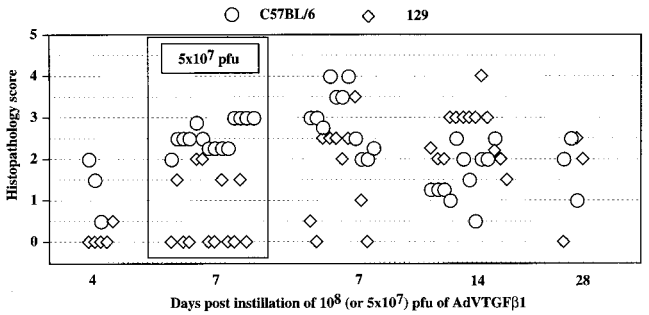

Figure 5. Histopathology scores of C57BL/6 and 129 mice after exposure to AdVTGFβ_1. Severity of disease pathology was quantified as described in Figure 2 and in MATERIALS AND METHODS. Diffuse interstitial inflammation and fibrogenesis became obvious in the C57 mice at Day 4, and was clearly present by 7 d after treatment, with diminution of the disease by the 28-d time period. By 7 d, at 5×10^7 pfu, 8 of the 13 129 mice exhibited no evidence of disease (score of 0), and at 10^8 pfu, 4 of 11 129 mice were essentially normal (score of 1 or less). At both concentrations of virus all 25 C57 mice had scores of 2 or greater at 7 d. By Day 14, all 129 mice exhibited scores greater than 1.

fibrogenesis in the lungs of two mouse strains. The 129 strain appeared to develop less asbestos-induced disease through the 1-mo period studied in our earlier work (19), and here as well, at the 48-h postexposure time point selected for a closer, quantitative comparison. When the inbred 129 mice were crossed with the C57 strain, an F1 generation was produced that exhibited a broad range of histopathologic responses to asbestos exposure (Figure 2). If there were a gene or closely related cluster of genes that is responsible for this apparent protected phenotype, backcrossing the F1 mice to the 129 inbred animals should separate out a subset of mice with the protected phenotype. This was the case inasmuch as 26% of these offspring did not develop asbestos-induced lung lesions (Figure 2).

It is obvious that we do not know what this gene or gene cluster might be. It will take a continued effort using the tissues and RNA samples we have preserved from each animal to sort out candidate genes that might allow a test of the hypothesis. Because we and a number of other investigators have been pursuing a "growth factor hypothesis," it is reasonable to suggest that candidate genes could be related to expression of peptide growth factors and/or to their specific receptors. For example, we have shown that primary lung fibroblasts from the 129 strain respond less to growth

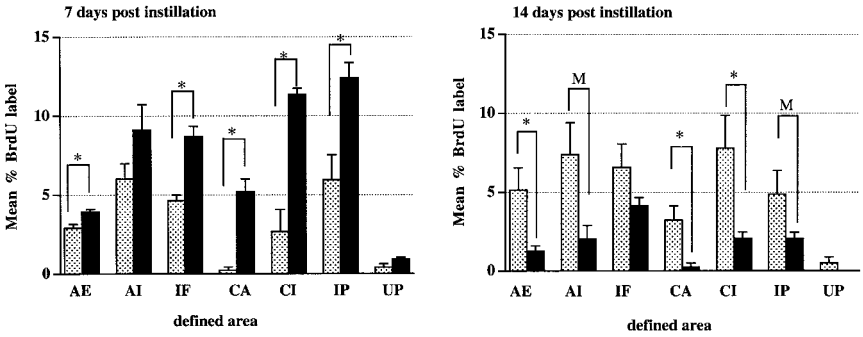

Figure 6. BrdU incorporation in AdVTGFβ_1-instilled C57BL/6 and 129 mice. At 7 and 14 d after treatment, BrdU-positive cells were quantitated by immunohistochemical staining and visualization using light microscopy at ×400 magnification. C57 mice exhibited significantly more proliferation at every anatomic region studied at 7 d. By 14 d, the 129 mice appeared to have more disease. The airway epithelium at both time points and in both strains exhibited a reduced proliferative response compared with the interstitium. All mice that received the control virus had mean % values between 0.00 and 0.24. AE, alveolar epithelium; AI, alveolar interstitium; IF, interstitial foci; CA, cross-section of airway epithelium; CI, cross-section of airway interstitium; IP, involved parenchyma; UP, uninvolved parenchyma. *$P < 0.05$ (comparison between the two strains); M = marginally significant.

factors such as TNF-α, PDGF, and TGF-β; the cells grow more slowly and produce less collagen *in vitro* than comparable cells from C57 mice, and these cells also appear to exhibit reduced expression of the RNA and protein that codes for the PDGF-α receptor (20, 27).

In the work presented here, we have asked if the differential response to growth factors is apparent *in vivo* in the 129 and C57 inbred mouse strains. The comparison using TGF-β was initiated for several reasons: (*i*) TGF-β is the most potent inducer of extracellular matrix components yet described (29, 30); (*ii*) TGF-β is expressed at sites of initial lung injury in our asbestos model (9, 28), other models of IPF appear to involve TGF-β to varying degrees (3, 4), and TGF-β has been identified in association with human IPF (29); and (*iii*) we have shown that an adenovirus vector transducing expression of active TGF-β causes dose-dependent interstitial fibrogenesis in mice (26), including fibrogenic-resistant TNF-α-receptor knockout mice (15).

TGF-β overexpression through the vector demonstrated the same principle as observed in the asbestos-exposed mice, i.e., that the 129 mouse strain exhibits an early, protected phenotype in a proportion of the mice. We had shown previously (26) that increasing concentrations of the viral vector transduce concomitant increases in the amount of TGF-β_1 released in C57 mice. We used this principle here, showing that at three concentrations of virus (5×10^7, 10^8, and 10^9 pfu), the 129 mice had a significantly lower mean histopathology score, and a number of individual animals exhibited no disease at 4 and 7 d after exposure (Figure 5). The fact that disease severity was reversed at 14 d in this model is unexplained at this time. However, this finding suggests that temporal factors may be involved in the observed "resistance to fibrogenesis." There also is the possibility that the TGF-β gene is expressed from the viral vector with different efficiency in the C57 versus 129 strains, although our experiments with instillations of an adenovirus expressing the gene for β-galactosidase (rAdVCMVLacZ) suggest that this is not the case, as both strains expressed the LacZ gene at comparable levels. However, given that this fibroproliferative process is caused by TGF-β_1, more of the growth factor protein would be expected in the lungs of the C57 mice early after treatment, and that is what was found at Day 4 (Figure 7). This would explain the worse disease in these C57 mice at a later time point (Day 7) after treatment (Figure 5). How early expression of TGF-β_1 mediates development of a fibroproliferative process through the subsequent 2 wk has yet to be established, although it is clear that TGF-β_1 is a potent chemotactic factor with a variety of effects on other cell types (29–31).

Active TGF-β_1 has been used in several laboratories to produce fibroproliferative lung disease (4). The active TGF-β_1 apparently induces expression of large amounts

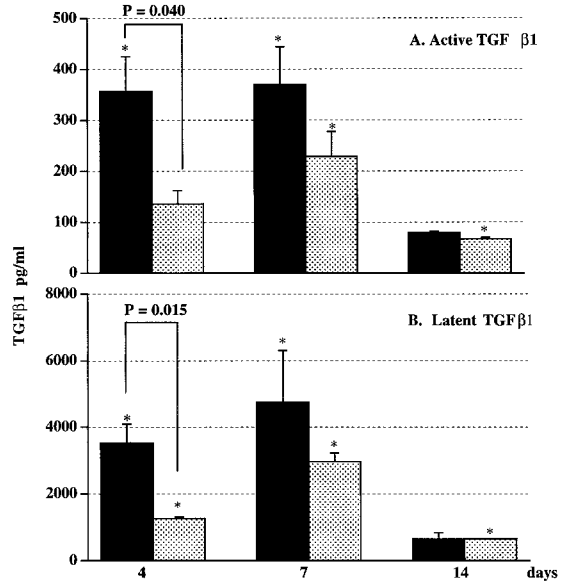

Figure 7. Quantitation of active and latent TGF-β_1 expression in the BAL fluid. TGF-β_1 protein in the lung after instillation of AV or AdVTGFβ_1 was measured by ELISA of the BAL fluid supernatant. Quantitation of active and latent TGF-β_1 showed significantly more protein at Day 4 from the lavage fluid of C57BL/6 mice than from the 129 mice ($P = 0.04$ for active TGF-β_1; $P = 0.015$ for latent TGF-β_1). At the 7-d time point, there was significantly more TGF-β_1 than in the control mice, but differences between the two strains did not reach statistical significance. * $P < 0.05$ compared with control.

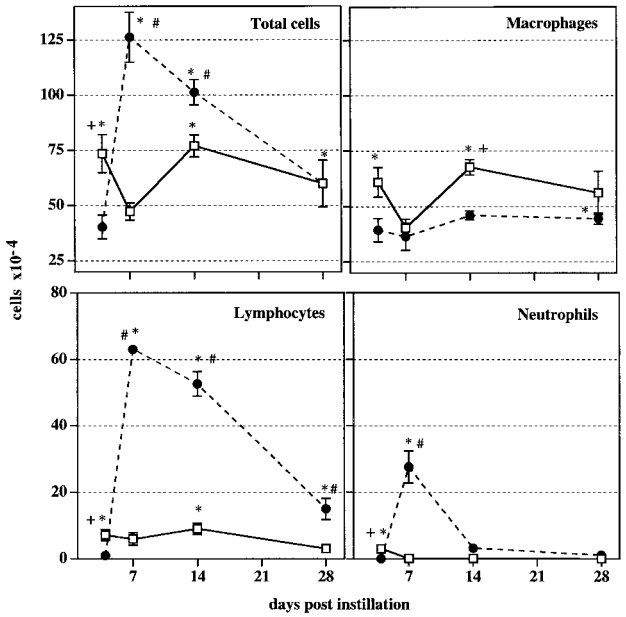

Figure 8. Cytologic evaluation of inflammatory cell accumulation in the lung. Cells collected from the BAL fluid were stained with Diff Quick for differential cell staining and cell counts were made on a light microscope at ×400 magnification. The time of peak inflammatory cell accumulation, 7 d for C57BL/6 and 14 d for 129 mice, was reflected by the cells recovered in BAL (*A*). When comparing individual cell types, the number of macrophages was consistently higher in 129 mice through Days 4–28 (*B*), the difference being statistically significant on Day 14 when the disease was worse in the 129 mice. On Day 7, neutrophils and lymphocytes were the dominating cell types in C57 mice (*C* and *D*), and the numbers were significantly higher compared with 129 mice. $^{*}P < 0.05$ over control; $^{\#}P < 0.05$ C57 higher compared with 129; $^{+}P < 0.05$ 129 higher compared with C57BL/6.

of endogenous latent TGF-β_1 by the mice, as shown here (Figure 7) and in previous work (4, 26). It is not difficult to envision the following: (*i*) rapid transduction of active TGF-β_1 within the first few days after treatment with the virus (Figure 7) (4, 26); (*ii*) activation of genes that code for latent TGF-β_1 and additional cytokines (4, 19, 26); (*iii*) recruitment of inflammatory cells and upregulation of matrix genes (Figure 8) (4, 19, 26); (*iv*) a delay or lack of activation of certain of these key genes (or lack of recognition of the TGF-β_1 signal, inactive or reduced receptor levels of TGF-β_1) in some individual animals of the 129 strain; and (*v*) failure to activate the latent TGF-β_1 in those animals which do not develop disease. The first three points have a great deal of support here and in the literature; the last two are hypothetical and should be tested as interesting possibilities for explaining the differences in disease development. In preliminary experiments performed with untreated C57 and 129 mice, the constitutive expression of TGF-β_1 receptors I and II and TNF-α receptors p55 and p75 appeared to be equal as measured by RNase protection assay of total lung RNA (personal observations).

A recent paper (32) has compared responses to lung injury in three strains of mice. The study shows microarray analysis of lung tissue from wild-type 129, C57 BL/6, and 129 αv β6 integrin knockout (KO) mice. There were clusters of genes that responded to bleomycin-induced lung injury in both the 129 and C57 strains, but these were minimally induced in the KO mice. It was fascinating to learn that the two 129 strains exhibited a delay in the proliferative response (32), even though the initial injury was similar, and all the animals eventually showed recovery of the injured airway epithelium. This is not unlike what we have reported here in two different models of lung injury. In the asbestos model, there is identical deposition of fibers (19), but there is reduced expression of the growth factors TGF-β and TNF-α (8, 9, 19). The mean reduction in lesion development reported in our earlier studies (19), probably was due to the reduced response to asbestos injury exhibited by a few individual animals in the 129 strain. These individuals can be more readily identified in the data shown here (Figure 2) where 26% of the F1 × 129 backcross mice exhibited no response to asbestos exposure. In the AdVTGF-β_1 model, there was a clear delay in the response of the 129 mice to TGF-β overexpression (Figure 5). At 4 d after exposure, the C57 mice produced more TGF-β_1, and at 7 d, many of them clearly had worse disease depending on the dose, i.e., at 5×10^7 pfu, 13 of 14 C57 mice, and at 10^8 pfu, 7 of 11 C57 mice had worse disease than the 129 mice (Figure 5). These consistent findings of a delayed and/or reduced response to injury in a subset of 129 mice both commercially-derived inbreds and genetically-manipulated backcrosses, along with similar results in primary mouse lung cells *in vitro* (20, 27), suggest that the 129 mouse strain can be an extremely useful model for defining "susceptibility" genes that control responses to lung injury.

Another recent paper describes attempts to assess gene expression in patients with sarcoidosis and IPF (33). Here the investigators showed a correlation between the occurrence of a polymorphism for IL-1α and sarcoidosis, thus demonstrating the potential of such an approach. This concept of looking for gene polymorphisms and susceptibility genes is becoming more prominent in the literature (34–36). If TGF-β, TNF-α, or other growth factors are central to the development of IPF, correlations with genes or related clusters could offer a key to understanding the mechanisms involved. It will be essential for us to determine whether or not similar gene arrays are found in 129 mice which fail to develop inflammation and fibrosis, i.e., those mice which are backcrossed and protected from asbestos (Figure 2) or those wild-type 129 mice that fail to develop TGF-β_1–induced IPF (Figure 5).

In conclusion, in the 129 mouse strain used in our studies presented here, it appears that a gene or gene cluster segregates sufficiently to control a degree of susceptibility to asbestos disease in some of the animals. Whether or not this is the same genetic influence that reduces the initial response to TGF-β expression in some of the inbred 129 mice is not known at this time. The gene and its products remain unknown, but should be identified and could offer important new targets for therapy.

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