Transfer of Tumor Necrosis Factor- α to Rat Lung Induces Severe Pulmonary Inflammation and Patchy Interstitial Fibrogenesis with Induction of Transforming Growth Factor- β 1 and Myofibroblasts

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Tumor necrosis factor- α is up-regulated in a variety of different human immune-inflammatory and fibrotic pulmonary pathologies. However, its precise role in these pathologies and, in particular, the mechanism(s) by which it may induce fibrogenesis are not yet elucidated. Using a replication-deficient adenovirus to transfer the cDNA of tumor necrosis factor- α to rat lung, we have been able to study the effect of transient but prolonged (7 to 10 days) overexpression of tumor necrosis factor- α in normal adult pulmonary tissue. We have demonstrated that local overexpression resulted in severe pulmonary inflammation with significant increases in neutrophils, macrophages, and lymphocytes and, to a lesser extent, eosinophils, with a peak at day 7. By day 14, the inflammatory cell accumulation had declined, and fibrogenesis became evident, with fibroblast accumulation and deposition of extracellular matrix proteins. Fibrotic changes were patchy but persisted to beyond day 64. To elucidate the mechanism underlying this fibrogenesis, we examined bronchoalveolar fluids for the presence of the fibrogenic cytokine transforming growth factor- β 1 and tissues for induction of α -smooth muscle actin-rich myofibroblasts. Transforming growth factor- β 1 was transiently elevated from day 7 (peak at day 14) immediately preceding the onset of fibrogenesis. Furthermore, there was a striking accumulation of myofibroblasts from day 7, with the most extensive and intense immunostaining at day 14, ie, coincident with the up-regulation of transforming growth factor- β 1 and onset of fibrogenesis. Thus, we have provided a model of tumor necrosis factor- α -mediated pulmonary inflammation and fibrosis in normal adult lung, and we suggest that the fibrogenesis may be mediated by the secondary up-regulation of trans-

forming growth factor- β 1 and induction of pulmonary myofibroblasts. (Am J Pathol 1998, 153:825–832)

Tumor necrosis factor- α (TNF- α) is a pleiotropic cytokine. It has been detected in a variety of human pulmonary diseases, but its role in these pathologies is not well understood. Based on its *in vitro* effects on leukocyte activation, adhesion molecule expression, and endothelial cell biology, it is also likely to have proinflammatory activities *in vivo*.¹ In addition, it may have fibrogenic potential, given that it is both mitogenic and chemotactic for fibroblasts,² although its effects on collagen gene expression are inhibitory.³ Clearly, however, up-regulation of TNF- α *in vivo* does not always result in the same biological outcome. In particular, TNF- α is detected in many inflammatory and immune diseases that resolve without tissue fibrosis,^{1,4} yet in other pathologies TNF- α up-regulation is associated with fibrotic sequelae.^{5,6} Whether these outcomes depend directly on TNF- α or indirectly on induction of other bioactive molecules, particularly the cytokines and growth factors, from both immune-inflammatory and structural cells remains unclear.

To investigate the functional activities of local pulmonary overexpression of TNF- α in lung, we have chosen to exploit a transient gene transfer approach using a replication-deficient recombinant adenovirus vector to transfer the cDNA of murine TNF- α to the respiratory epithelium. Using similar cytokine-expressing adenovectors under control of high-efficiency constitutive promoters, we and others have shown that after intratracheal administration, the vectors infect respiratory epithelium and result in local and transient, but sustained, production of their transgene protein in the background of a normally developed adult lung.^{7–9} This is likely to mimic the type of cytokine up-regulation seen in human immune and inflammatory pulmonary pathologies.

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Using this gene transfer approach we have been able to demonstrate that transient local overexpression of high levels of TNF- α in adult rat lung results in intense pulmonary accumulation of neutrophils, macrophages, and lymphocytes and, to a lesser extent, eosinophils over 2 to 10 days without gross distortion of the lung architecture. This is followed by the development of patchy interstitial fibrogenesis, associated with significant increases in transforming growth factor- β 1 (TGF- β 1) protein and accumulation of myofibroblasts.

Materials and Methods

Adenoviral Vectors and Administration to Rat Lung

A replication-deficient recombinant adenovirus expressing full-length murine TNF- α cDNA under control of an efficient murine cytomegalovirus promoter and with the interferon- γ -secretory leader sequence was used to locally overexpress TNF- α in the lung. Its construction and demonstration of release of the secreted form of TNF- α have been previously described.¹⁰ This vector or a control vector with identical deletions (AdDL70-3)¹¹ at a dose of 10^9 plaque-forming units in 300 μ l of phosphate-buffered saline was instilled intratracheally to anesthetized Sprague Dawley rats weighing 220 to 280 g. All animal experiments were approved by the McMaster University Animal Ethics Committee. Rats were provided with food and water *ad libitum* and were examined at 1, 3, 7, 14, 21, 28, or 64 days. Blood was taken from the abdominal aorta, and bronchoalveolar lavage (BAL) was performed as previously described.⁸ BAL total cell counts were determined using a hemacytometer. Differential cell counts were performed on cytopins stained with Diff-Quik (Baxter, McGaw Park, IL) by randomly counting 500 cells/cytospin. Three to five animals per group were evaluated. BAL fluid and sera were stored at -20°C until TGF- β 1 and TNF- α levels were determined in BAL fluid and serum as described below.

TNF- α and TGF- β Measurements

Concentrations of TNF- α in BAL fluid and serum were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit specific for murine and rat TNF- α (R&D Systems, Minneapolis, MN) with a sensitivity of 5 pg/ml. TGF- β 1 in BAL fluid was measured using a commercial human TGF- β 1 ELISA kit that efficiently detects rat TGF- β 1 protein due to the high homology of TGF- β 1 across species (R&D Systems). The assay detects only the active form of TGF- β 1, and samples were activated before measurement according to the manufacturer's instructions. The sensitivity of this kit is 5 pg/ml. Analysis was performed on samples from three to six animals per group.

Lung Fixation and Histological Examination

Lungs were removed and fixed by perfusion with neutral buffered formaldehyde before routine processing and paraffin embedding. Multiple sections from each lobe were stained with hematoxylin and eosin or Elastic van Gieson, a specific histochemical stain for collagen and elastin. Lungs from three to six rats per time point were examined.

Immunohistochemical Staining for α -Smooth Muscle Actin

Lung tissue sections were deparaffinized, and endogenous peroxidase was blocked. Sections were then treated with blocking goat serum for 30 minutes and were incubated for 16 hours with a monoclonal anti- α -smooth muscle actin (α -SMA) antibody (Sigma Chemical Co., St. Louis, MO) at a dilution of 1:100. Control sections were treated with control mouse immunoglobulin G. Sections were then incubated for 15 minutes with biotinylated goat anti-mouse immunoglobulin (Histostain-SPTM Bulk Kit; Zymed Laboratories, Inc., San Francisco, CA) and treated for 10 minutes in streptavidin-peroxidase conjugate. Finally, they were placed in a substrate chromogen mixture, and color was allowed to develop for 15 minutes before counterstaining with Mayer's hematoxylin.

Data Analysis

Data are expressed as means $\pm$ standard error of the mean (SEM). Statistical analysis was performed using an unpaired *t*-test. The difference was considered statistically significant when $P < 0.05$.

Results

TNF- α Protein Expression in Vivo after TNF- α Gene Transfer to the Lung

To assess the effects of local overexpression of TNF- α in lung tissue, AdTNF- α or control virus AdDL70-3 was injected intratracheally into rat lung. TNF- α protein expression in lung was then examined at various time points by assaying BAL fluid by ELISA. In rats infected with AdTNF- α , TNF- α protein expression was high between days 1 and 7, peaked at day 3 (154.9 ± 45.3 ng/ml), and rapidly declined by day 14 (Figure 1). In comparison, levels of TNF- α induced by the control virus were very low, with a peak also at day 3 (0.14 ± 0.12 ng/ml) (data not shown). These differences between AdTNF- α - and AdDL70-3-infected rats were highly significant $P < 0.0001$ at day 3 and $P = 0.0062$ at day 7. To confirm that the transgene product was largely compartmentalized in the lung, sera were taken at various time points and assayed by ELISA for TNF- α protein. TNF- α was not detected in sera of any rats infected with control virus. In rats infected with AdTNF- α , TNF- α protein was detected

Figure 1. TNF- α protein concentration in BAL fluid. BAL fluid samples were collected from rats receiving AdTNF- α or control virus at various time points and were assayed for TNF- α by ELISA. Control virus resulted in production of 0.14 ± 0.12 ng/ml TNF- α (data not graphed). Results shown are for AdTNF- α -infected rats and are expressed as means $\pm$ SEM from three to six animals/time point. * $P < 0.0001$ and ** $P = 0.0062$, respectively, between AdTNF- α and control virus.

in animals only at days 1 and 3, and levels were about 100- to 300-fold lower than in the BAL fluid, ie, 0.21 ± 0.051 ng/ml at day 1 and 0.53 ± 0.06 ng/ml at day 3.

Cellular Changes in the BAL Fluid after TNF- α Gene Transfer to the Lung

To assess the cellular responses after overexpression of TNF- α in the lung, total and differential cell counts in BAL fluid were quantitated at a variety of time points. Infection with the control vector resulted in low levels of neutrophilia at day 1 and, subsequently, a small increase in the number of macrophages and lymphocytes at day 7 (Figure 2A/B). In contrast, infection with AdTNF- α resulted in a dramatic accumulation of neutrophils, macrophages (Figure 2A), and lymphocytes in the BAL fluid (Figure 2B). Cell accumulation was evident from day 3 but was most significant at day 7, when, compared with control vector-treated rats, AdTNF- α -treated rats demonstrated a 39-fold increase in neutrophils, a 6-fold increase in macrophages, and a 17-fold increase in lymphocytes. There was also a small increase in eosinophils. The cellular accumulation resolved thereafter. Neutrophil and macrophage numbers returned toward those observed in rats infected with control virus by day 14, and lymphocytes numbers returned to control levels by day 21.

Figure 2. Effects of TNF- α overexpression on macrophage and neutrophil accumulation (A) and lymphocyte and eosinophil accumulation (B) in BAL fluid. Rats were infected intratracheally with AdTNF- α or control virus, and BAL fluid was recovered as described in text. Differential cell counts were then determined on Diff-Quik-stained cytopins. Results are expressed as means $\pm$ SEM from three to five animals/time point.

Pulmonary Tissue Responses after TNF- α Gene Transfer to the Lung

Rats infected with control virus appeared healthy, and microscopic examination showed some minor inflammatory changes with a few neutrophils and mononuclear cells in the perivascular and peribronchial areas at days 1, 3, and 7. These inflammatory changes did not extend into the alveolar spaces or septae beyond, and they rapidly cleared with no evidence of tissue remodeling or fibrosis, similar to the minimal changes seen with a second control vector expressing the marker gene β -galactosidase.⁸ In contrast, rats receiving AdTNF- α lost weight and appeared lethargic. Macroscopically, areas of consolidation were evident at early time points. Microscopic examination of their lungs revealed widespread and severe inflammatory changes beginning 24 hours after in-

Figure 3. Histopathological effects induced by overexpression of AdTNF- α at various time points. **A, B, C,** and **E** are stained with hematoxylin and eosin; **D** is stained with Elastic van Gieson (collagen stained red and elastin black). Cells are shown on day 3 (**A**), day 7 (**B**), day 14 (**C** and **D**), and day 64 (**E**) after infection. Magnifications: $\times 160$ (**A, B,** and **E**) and $\times 250$ (**C** and **D**). **Arrows** show fibrogenic response in the interstitium. **Arrowhead** shows patchy scarring and distorted architecture in the interstitium. **b**, bronchial structure; **v**, vascular structure.

fection, when there was accumulation of neutrophils in the alveolar spaces and of neutrophils and mononuclear cells in the interstitium, particularly in perivascular and peribronchial areas (data not shown). These changes were much more severe than in control vector-treated rats. By day 3, accumulation of inflammatory cells was more pronounced (Figure 3A), and by day 7, in keeping with the kinetics of cell accumulation in the BAL fluid, there was intense tissue inflammation with dramatic accumulation of neutrophils and mononuclear cells, particularly in perivascular and peribronchial areas, but extending widely throughout the parenchyma right up to the pleural surface (Figure 3B). The alveolar septae were expanded by infiltrated inflammatory cells, but the general tissue architecture was maintained. Only a few eo-

sinophils were observed. By day 14, the tissue neutrophilia had resolved while there was still accumulation of perivascular and peribronchial mononuclear cells, although this was much less severe than at day 7. However, at this time point, there was thickening of some alveolar walls and emergence of areas of interstitial fibrosis with accumulation of fibroblasts and destruction of normal tissue architecture (Figure 3C). Deposition of disorganized collagen and elastic fibers was evident on Elastic van Gieson staining (Figure 3D). Inflammation had greatly declined by day 21, but some patchy fibrotic changes persisted, with areas of fibrosis scattered throughout the parenchyma, sometimes reaching the pleural surface. These fibrotic changes persisted up to day 64 (Figure 3E), when there was deposition of more

Figure 4. TGF- β 1 concentration in BAL fluid. BAL samples were collected from rats receiving AdTNF- α or control virus (AdDL70-3) at various time points and were assayed for TGF- β 1 by ELISA. Results are expressed as means $\pm$ SEM from three to six animals/time point. * $P = 0.0057$ between AdTNF- α and control virus.

organized collagen and elastin fibers. Inflammatory changes had largely resolved by this point.

Examination of TGF- β 1 Production after TNF- α Gene Transfer to the Lung

To help address the mechanism of TNF- α -induced interstitial fibrosis, we examined the content of TGF- β 1 in BAL fluid by ELISA. We and others have shown TGF- β 1 to be a key fibrogenic cytokine in the lung.^{7,12} Increased levels of TGF- β 1 were evident in AdTNF- α -treated rats from day 3 with a peak at day 14 ($P = 0.0057$, AdTNF- α compared with control) and a decrease back toward control levels by day 64 (Figure 4). No such increase in TGF- β 1 was observed in control vector-infected rats.

Immunolocalization of Myofibroblasts after TNF- α Gene Transfer to the Lung

The myofibroblast is a fibroblast-like cell characterized by the expression of α -SMA¹³ and is thought to be important in fibrogenesis and wound healing.¹⁴ To assess whether TNF- α overexpression resulted in the generation of myofibroblasts during the fibrogenic process, we examined lung tissues for induction of α -SMA-expressing myofibroblasts using a monoclonal antibody directed against α -SMA. In control vector lungs and in lungs of AdTNF- α -infected rats, immunostaining of bronchial wall and vascular smooth muscle was observed as expected. There was no other immunostaining in control virus-treated animals at any time points. In AdTNF- α -treated rats, there was no increase in α -SMA immunostaining above control virus-infected animals at day 3 (Figure 5A); however, by day 7, immunostaining of fibroblastic cells

was evident widely throughout the parenchyma (Figure 5B). By day 14, when fibrogenesis was evident, there was intense and extensive immunostaining of fibroblastic cells in fibrotic foci within the interstitium (Figure 5C). Immunostaining within these fibrotic areas persisted up to day 64, although it was not as extensive or intense as it had been on day 14 (Figure 5D). The fibroblast-like cells staining for α -SMA also immunostained with an antibody directed against vimentin. This confirmed that these cells were myofibroblasts and not smooth muscle cells.

Discussion

TNF- α is an "early-wave" alarm-type cytokine. It appears to be up-regulated soon after injury in a number of tissues including lung, and increased TNF- α expression has certainly been documented in a wide variety of different human pulmonary diseases and animal models. However, our understanding of its precise role, particularly in the process of pulmonary fibrogenesis, is poorly understood. An effective strategy of assessing the role of any cytokine *in vivo* is to locally overexpress it in a tissue-specific manner, and there are several ways to achieve this. Administration of recombinant cytokines is one possibility. Using this approach, Ulich et al¹⁵ administered recombinant TNF- α to rat lung and demonstrated induction of moderate neutrophilia, which resolved within 48 hours, leaving no evidence of permanent tissue damage. An alternative strategy is to develop a tissue-specific transgenic animal model, and using this approach, Miyazaki et al¹⁶ reported their findings in a transgenic model in which TNF- α gene expression was driven from the lung-specific SP-C promoter. In contrast to the recombinant protein experiments, these mice developed an early lymphocytic alveolitis, followed later by a fibrogenic response. Although both of these approaches provide useful information on the *in vivo* biological effects of TNF- α , they may not accurately reflect the type of overexpression seen in adult human disease, in which TNF- α up-regulation is likely to be more prolonged than in experiments using recombinant proteins, but shorter than the lifelong overexpression of genetic transgenics. In addition, unlike the permanent transgenic models in which abnormal lung development can occur as a consequence of transgene overexpression *in utero*, most adult lung disease occurs in the background of a normally developed lung.^{16,17} To overcome some of these problems and to examine the role of TNF- α overexpression in normal adult lung, we chose a transient transgenic approach using a replication-deficient adenovirus.¹⁰ We demonstrated that delivery of the adenoviral gene transfer vector resulted in high levels of transgene overexpression with a peak at day 3 and a rapid decline after 7 days. This induced rapid and intense accumulation of neutrophils, macrophages, and lymphocytes from 24 hours. Inflammation in both BAL fluid and tissue was maximal at day 7, ie, shortly after the peak of transgene protein expression. Inflammation thereafter started to resolve, although inflammatory changes were still evident at days

Figure 5. α -SMA-positive cells in lung tissue from rats infected with AdTNF- α . Lungs were fixed and processed for immunohistochemical staining using a monoclonal antibody directed against α -SMA. **A:** Cells on day 3 after infection. Immunoreactivity is primarily localized to the smooth muscle layer (arrow) around a bronchial structure (b) and a vessel (v). **B and C:** Cells on days 7 and 14 after infection, respectively. Arrowheads in **C** indicate intense immunostaining of fibroblastic cells in fibrotic foci. **D:** Cells on day 64. Arrows indicate less intense immunostaining in a fibrotic area. Magnification: $\times 250$ (**A** to **D**).

21 and 28, long after transgenic protein was no longer detectable. The inflammatory changes were most severe in the peribronchial areas, likely reflecting the high concentrations of transgene protein in these areas. However, these changes also spread throughout the parenchyma up to the pleural surface, either as a result of diffusion of the cytokine itself, or more likely by TNF- α -mediated induction of other inflammatory cytokines such as interleukin-8, granulocyte-macrophage colony-stimulating factor, or macrophage chemotactic protein-1 from adjacent cells in a paracrine fashion.^{18,19} The neutrophilia in this model was more severe than in either the recombinant protein or transgenic models, perhaps because of higher local tissue concentrations of TNF- α . Lymphocytic accumulation was a notable feature in the transgenic model, as in the present study, but increases in the number of macrophages seem specific to our model. The mechanisms of this TNF- α -mediated accumulation of neutrophils, lymphocytes, macrophages, and eosinophils are likely to involve both direct effects of TNF- α itself on regulation of adhesion molecule expression and induction of other cytokines and growth factors capable of mediating leukocyte chemotaxis and survival.¹ After the intense inflammation in the tissue, there was evidence of a fibrogenic response. This was evident from day 14,

when there was accumulation of fibroblasts and deposition of both collagen and elastin. The destruction of normal lung architecture and the persistence of these changes to day 64 likely indicate that this fibrogenesis is, at least in part, irreversible. Certainly, TNF- α has been shown to be up-regulated in a number of fibrotic human and animal pulmonary pathologies,^{5,6,20,21} and there is evidence that inhibiting early TNF- α expression with either anti-TNF- α antibodies or TNF- α antagonists inhibits fibrogenesis.^{22,23} Equally, however, TNF- α up-regulation has been documented in other inflammatory and immune lung pathologies in which normal lung repair processes ensue without evidence of fibrotic reactions.¹ This raises the question of the precise role of TNF- α *in vivo* during pulmonary fibrogenesis and whether TNF- α may be acting indirectly through up-regulation of other fibrogenic molecules during pathological processes with a fibrotic sequela. In particular, it appears that TGF- β 1 is up-regulated in many of the pathologies in which TNF- α is associated with fibrosis but not in those where TNF- α expression results in immune-inflammatory damage without a fibrotic outcome.^{5,24,25} To address this hypothesis, we examined the AdTNF- α -infected animals for up-regulation of TGF- β 1 protein and found increased protein synthesis from day 3, with a peak around day 7 to 14,

immediately preceding the onset of fibrogenesis. To further investigate the mechanism of the fibrogenesis in this model, we then examined tissue for induction of α -SMA-expressing myofibroblasts. Myofibroblasts have been demonstrated in a variety of fibrotic and repairing tissues^{14,26} and have been shown to be the major cells producing procollagen mRNA in the bleomycin model of fibrosis.²⁷ In the AdTNF- α -treated rats, aside from the expected immunopositive bronchial and vascular smooth muscle, we found α -SMA and vimentin-immunopositive fibroblast-like cells from day 7 scattered widely throughout the parenchyma. By day 14, there was intense immunostaining within fibroblast-like cells in the fibrotic foci, and staining persisted to day 64 although, its intensity and extent were lessened. Interestingly, in the studies of Rubbia-Brandt et al,²⁸ in which TNF- α was administered subcutaneously by osmotic minipump, fibroblast accumulation occurred, but there was no evidence of induction of myofibroblasts and minimal collagen deposition, suggesting that TNF- α overexpression alone is insufficient to generate myofibroblasts.²⁸ In contrast, overexpression of TGF- β 1 in lung or skin results in induction of many α -SMA-rich myofibroblasts and extensive collagen deposition.^{7,29} These data suggest that in the present model, induction of myofibroblasts may be secondary to the increased TGF- β 1 protein rather than to a direct effect of TNF- α . Certainly, the temporal association of increased TGF- β 1 and induction of myofibroblasts at days 7 to 14 would support this concept. Interestingly, in the transgenic study of Miyazaki et al,¹⁶ no evidence of TGF- β gene activation was found, and there was no induction of myofibroblasts. However, only late time points, after fibrogenesis was established, were examined. The cellular origin of the TGF- β 1 in this current model is under investigation. In other fibrogenic models, macrophages,^{30,31} eosinophils,³² and tissue structural cells¹ have all been shown to be important sources, and it is therefore noteworthy that macrophage and eosinophil numbers are increased early in our model. It is interesting to note that based on our own studies of adenovector-mediated overexpression of other fibrogenic cytokines, including TGF- β 1,⁷ granulocyte-macrophage colony-stimulating factor,³³ and now TNF- α , there appears to be a correlation between the levels of TGF- β 1, the extent of myofibroblast induction, and fibrosis. Specifically, in the TGF- β 1 model, TGF- β 1 levels are very high, myofibroblasts are very numerous, and fibrosis is severe, but in the TNF- α model, levels of TGF- β 1 are lower; myofibroblast induction is less than in the TGF- β 1 model, especially at later time points; and fibrosis is correspondingly less severe. The granulocyte-macrophage colony-stimulating factor model lies intermediately between the other models in all three parameters: TGF- β 1 induction, myofibroblast presence, and fibrosis.³⁴

In conclusion, our study has provided evidence that in normal adult lung, transient overexpression of TNF- α results in intense but transient inflammation and patchy interstitial fibrosis associated with induction of TGF- β 1 and myofibroblasts.

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