Adenovector-mediated Gene Transfer of Active Transforming Growth Factor-β1 Induces Prolonged Severe Fibrosis in Rat Lung

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Abstract

Transforming growth factor (TGF)-β1 has been implicated in the pathogenesis of fibrosis based upon its matrix-inducing effects on stromal cells in vitro, and studies demonstrating increased expression of total TGF-β1 in fibrotic tissues from a variety of organs. The precise role in vivo of this cytokine in both its latent and active forms, however, remains unclear. Using replication-deficient adenovirus vectors to transfer the cDNA of porcine TGF-β1 to rat lung, we have been able to study the effect of TGF-β1 protein in the respiratory tract directly. We have demonstrated that transient overexpression of active, but not latent, TGF-β1 resulted in prolonged and severe interstitial and pleural fibrosis characterized by extensive deposition of the extracellular matrix (ECM) proteins collagen, fibronectin, and elastin, and by emergence of cells with the myofibroblast phenotype. These results illustrate the role of TGF-β1 and the importance of its activation in the pulmonary fibrotic process, and suggest that targeting active TGF-β1 and steps involved in TGF-β1 activation are likely to be valuable antifibrogenic therapeutic strategies. This new and versatile model of pulmonary fibrosis can be used to study such therapies. (*J. Clin. Invest.* 1997. 100:768–776.) Key words: gene transfer • cytokine • fibrotic • extracellular matrix • pulmonary

Introduction

The pathogenesis of pulmonary fibrosis is poorly understood. Recent studies, however, have indicated that a number of cytokines, particularly TGF-β, are important. There are three isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) in mammals, that have similar and wide-ranging effects on cell proliferation, differentiation, and migration (1). The TGF-β1 isoform has been most widely studied in the context of fibrogenesis, and has a number of actions on cells in vitro that are relevant to the fibrotic process. It is mitogenic and chemotactic for fibroblasts and monocytes/macrophages, and promotes accumulation of extracellular matrix (ECM)[1] proteins by increasing their synthesis while inhibiting production of matrix-degrading enzymes (2). TGF-β1 is produced naturally as a latent 390-amino acid dimeric precursor (3) that is converted into a mature bioactive 112–amino acid dimer after cleavage and dissociation of the amino-terminal portion, termed the latency associated peptide (LAP) (4, 5). This cleavage and dissociation can be readily achieved in vitro nonphysiologically by extremes of pH, temperature, and chaotropic agents (6). In vivo, however, the activation mechanisms have not yet been fully elucidated, although thrombospondin (7) and the proteases plasmin and cathepsin have been implicated (8).

In vivo, a role for TGF-β1 in pulmonary fibrosis is supported by studies demonstrating increased TGF-β1 gene expression and protein secretion in the lungs of animals (9) and humans (10, 11) with fibrotic diseases. Furthermore, a temporal and contiguous association has been demonstrated between such TGF-β1 expression and ECM gene expression (12). Most studies to date have examined only total TGF-β1 expression, and have not differentiated latent protein from biologically active TGF-β1. It has therefore been difficult to address the functional importance of TGF-β1 and its activation in these diseased tissues.

Recombinant adenoviral vectors provide an efficient method of overexpressing cytokine genes in a tissue-specific manner. When introduced intratracheally, the vectors readily infect respiratory epithelium (13) and result in production of functionally active transgene protein. Using this approach, we have previously investigated the functional activities of a variety of different cytokines, chemokines, and growth factors (13–15). Similarly, in this study we have achieved a transient and tissue-specific overexpression of TGF-β1 protein similar to the pattern of local cytokine expression likely occurring in humans and animals during fibrogenesis by using adenoviral vectors expressing TGF-β1. We first overexpressed the latent form of TGF-β1. This overexpression allowed for assessment of the tissue responses produced, and the levels of in situ activation achieved. We then overexpressed a protein with two mutations in the LAP portion of the molecule, resulting in production of spontaneously active protein, thus allowing investigation of the effects of overexpression of intrinsically active TGF-β1. We have provided evidence for the first time that overexpres-

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1. *Abbreviations used in this paper:* BAL, bronchoalveolar lavage; ECM, extracellular matrix; EVG, Elastic van Gieson; IT, intratracheally; LAP, latency associated peptide; SMA, smooth muscle actin.

sion of the active, but not latent, TGF-β1 in rat lung results in prolonged and severe interstitial and pleural fibrosis.

Methods

Construction of recombinant adenoviruses. Two recombinant adenoviruses were constructed (see Fig. 1, *A* and *B*). Both contained the cDNA of the coding region of full-length porcine TGF-β1. The first (AdTGF-β1) expressed latent (inactive) protein, while the second (AdTGF-$\beta1^{223/225}$) contained a mutation of cysteine to serine at positions 223 and 225, rendering the expressed TGF-β1 biologically active (16). 1,500-bp (TGF-β1) and 1,100-bp (TGF-$\beta1^{223/225}$) cDNA fragments were isolated from the Okayama and Berg Cloning Vector and plasmid pPK9A (a kind gift from Dr. Anita B. Roberts, Laboratory of Chemoprevention, National Institutes of Health, Bethesda, Maryland), respectively, by digestion with the restriction enzymes KpN and BamH1 (TGF-β1) and BglII (TGF-$\beta1^{223/225}$) (New England Biolabs, Inc., Beverly, MA). These fragments were subcloned using T4 ligase (New England Biolabs, Inc.) into the shuttle plasmid pACCMV. PLP.ASR(+) (kindly provided by Dr. Bob Gerard, Department of Cardiology, Howard Hughes Medical Institute, University of Texas), which contains the left end of the human adenovirus type 5 genome (0–17 mU) and a human cytomegalovirus promoter, a multicloning site, and an SV40 polyadenylation signal inserted into the E1 region. The resulting chimeric plasmids PACCMVTGF-β1 and PACCMVTGF-$\beta1^{223/225}$ were each cotransfected with an adenovirus derivative pBHG10 and PJM17, respectively, each of which has a deletion in the E1 region (17). After spontaneous homologous recombination between the plasmids, recombinant replication-deficient adenoviruses (AdTGF-β1 and AdTGF-$\beta1^{223/225}$) were rescued. A control virus AdDL70-3 was constructed as previously described (17).

Viruses were expanded, purified, and plaque-titered in 293 cells, as previously described (14).

In vitro characterization of the TGF-β1–expressing adenoviruses. Supernatants of 293 cells infected at a multiplicity of infection of 10 were assayed for TGF-β1 protein using a commercial human TGF-β1 ELISA kit (R & D Systems, Inc., Minneapolis, MN) which also detects porcine protein due to the high homology of TGF-β1 (sensitivity 5 pg/ml). This ELISA is receptor based, and only detects active TGF-β1. Direct measurement of samples detected levels of spontaneous active TGF-β1. Samples were also activated by acidification (18) to dissociate the LAP, and to detect total (active + latent) protein. Biological activity of protein from both the in vitro and in vivo experiments was confirmed using a murine HT-2 lymphocyte cell line as described by Tsang et al. (19).

Administration of recombinant adenoviruses to rat lung and transgene protein detection. 10^9 pfu of AdTGF-β1, AdTGF-$\beta1^{223/225}$, or control virus DL70-3 in 300 μl PBS was instilled intratracheally (IT) into anaesthetized male Sprague-Dawley rats weighing 250–275 g as previously described (14). Animals were examined at 1, 3, 7, 14, 21, 28, and 64 d. Blood was taken from the abdominal aorta, and bronchoalveolar lavage (BAL) was performed as previously described (14). Active and total TGF-β1 protein expression was assessed in the BAL and serum by both the ELISA and bioassay described above in three to five animals per time point.

Lung fixation and histological examination. Lungs were removed, and the right lung was 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 (EVG), a specific histochemical stain for collagen and elastin. Lungs from three to five animals per time point were examined. The left lung was weighed, snap frozen in liquid nitrogen, and stored at −70°C before assessment of the hydroxyproline content.

Lung hydroxyproline determination. This was performed according to established procedures and colorimetric measurement (20). Results were calculated as hydroxyproline content per wet weight lung tissue. Lungs from three to five animals per time point were examined in the hydroxyproline assay.

Immunohistochemical staining for α smooth muscle actin (SMA), fibronectin, and vimentin. Lung tissue sections were deparaffinized, and endogenous peroxidase was blocked. Sections to be stained for fibronectin were digested in 0.4% wt/vol pepsin in 1 M acetic acid, and sections to be stained for vimentin were treated with EDTA for 10 min. All sections were then treated with blocking goat serum for 30 min, and were incubated for 16 h with the primary antibody. The polyclonal rabbit antifibronectin antibody (DAKO Corp., Carpinteria, CA) was used at a dilution of 1:4,000. The monoclonal anti-αSMA antibody (Sigma Chemical Co., St Louis, MO) was used at a dilution of 1:100, and the monoclonal antivimentin antibody (Dako Corp.) was used at a dilution of 1:200. Some sections were incubated with either rabbit serum (Sigma Chemical Co.) or control mouse IgG as negative controls. Sections were then incubated for 15 min with biotinylated goat anti–rabbit or anti–mouse Ig (Histostain-SPTM Bulk Kit; Zymed Labs, Inc., S. San Francisco, CA), and incubated for 10 min in streptavidin peroxidase conjugate. Finally, they were placed in a substrate/chromogen mixture, and color was allowed to develop for 15 min before counterstaining with Mayer's hematoxylin.

Data analysis. Data were expressed as mean±SEM. Statistical analysis was performed using an unpaired *t* test. The difference was considered statistically significant when $P < 0.05$.

Results

Characterization of the AdTGF-β1 and AdTGF-$\beta1^{223/225}$ adenovirus constructs. To allow transient overexpression of either latent or active TGF-β1, we constructed two replication-deficient recombinant adenoviruses (Fig. 1, *A* and *B*) (21). The construct AdTGF-β1 produces latent protein due to the presence of a normally functioning LAP. In contrast, the AdTGF-$\beta1^{223/225}$ contains two point mutations in the LAP portion of the molecule, preventing the LAP from forming a homodimer and associating with the mature active TGF-β1, thus producing protein that is intrinsically active. Southern blot analysis of HindIII restriction digests of viral genomic DNA showed the TGF-β1 and TGF-$\beta1^{223/225}$ transgenes as 1.6- and 1.1-kb fragments, respectively, as expected (data not shown). Transgene protein production and secretion by cells infected with the recombinant viruses was verified by ELISA for TGF-β1 protein. This receptor-based ELISA detects only bioactive TGF-β1, and requires preactivation of samples by acidification to detect latent TGF-β1 by converting it to bioactive protein. The 293 cells infected with AdTGF-β1 for 24 h produced 27 ng/10^6 cells of latent (inactive, but acid-activatable) TGF-β1, and no spontaneously active TGF-β1, and AdTGF-$\beta1^{223/225}$ infection produced 15 ng/10^6 cells of spontaneously active TGF-β1 with an additional 28 ng/10^6 cells of latent protein. Control (DL70-3) virus infection induced no TGF-β1 production. The biological activity of the transgene product was confirmed by a T cell proliferation bioassay (data not shown).

Active and total TGF-β1 expression in vivo after TGF-β1 and TGF-$\beta1^{223/225}$ gene transfer to the lung. Analysis of bronchoalveolar fluids from rats infected IT with AdTGF-β1 revealed high levels of total (active and latent) TGF-β1 protein (Fig. 2 *A*). Expression was evident from day 1, peaked at day 7 (23.3 ng/ml), and continued for > 14 d. The majority of this protein was in the latent (inactive) form, measurable in the ELISA and bioassay only after acid activation. Total protein expression in these rats was statistically different from rats infected with control virus at day 3 ($P < 0.005$), and day 7 ($P <$

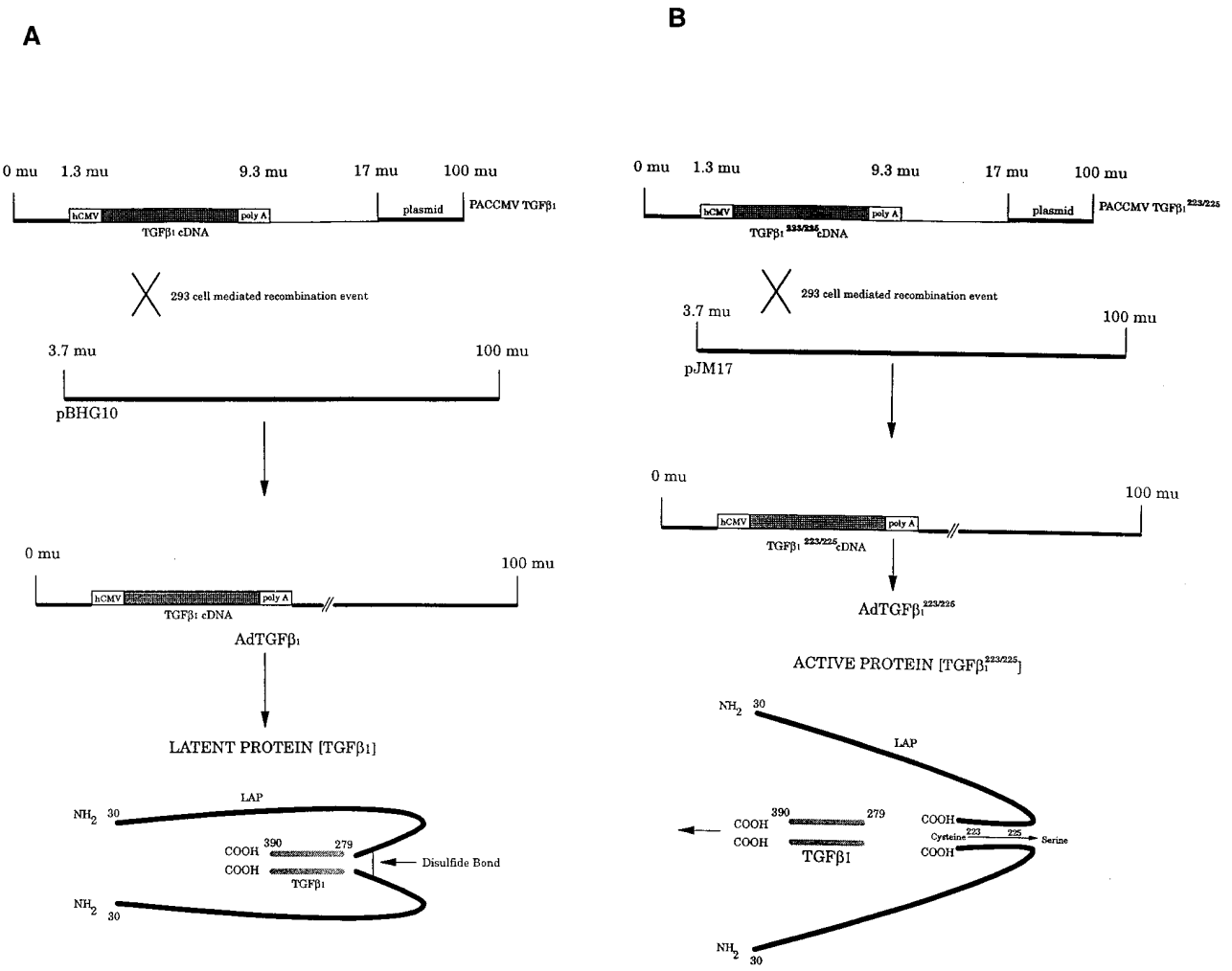


Figure 1. Construction of recombinant adenoviruses AdTGF-β1-(*A*) and AdTGF-β1$^{223/225}$ (*B*)-expressing porcine TGF-β1. The recombinant plasmids PACCMVTGF-β1 (*A*) and PACCMVTGF-β1$^{223/225}$ (*B*) were constructed by inserting the cDNA of TGF-β1 into the multicloning site of a shuttle vector pACCMV, which contains a cytomegalovirus promoter and an SV40-splicing junction/polyA signal. The recombinant adenoviruses were generated by homologous recombination after cotransfecting 293 cells with PACCMVTGF-β1 (*A*) or PACCMVTGF-β1$^{223/225}$ (*B*) and an adenovirus derivative pBHG10 (*A*) or PJM17 (*B*). The cDNA of the AdTGF-β1$^{223/225}$ construct contains point mutations of cysteines 223 and 225 to serines in the prepro portion of full-length TGF-β1 termed the LAP, which results in production of largely constitutively active TGF-β1 protein.

0.0005). The amount of spontaneously active TGF-β1 was low (111–470 pg/ml), but followed similar kinetics with peak activity at day 7. In contrast, analysis of the BAL fluid from rats infected IT with AdTGF-β1$^{223/225}$ demonstrated significantly higher levels of both active and total TGF-β1 (Fig. 2 *B*) than rats infected with control virus. Peak protein production was again at day 7. In addition to high levels of spontaneously active TGF-β1 (mean 13.1 ng/ml at day 7 [$P < 0.0005$]), there were also significant increases in production of latent TGF-β1 (mean 72.3 ng/ml at day 7 [$P < 0.0001$]). Infection of rats with control vector (AdDL70-3) resulted in either no, or only very low levels (< 300 pg/ml) of latent protein, and no spontaneously active protein.

There was no increase in active or latent TGF-β1 in the serum after IT delivery of either AdTGF-β1$^{223/225}$ or AdTGF-β1.

Pulmonary tissue responses after TGF-β1 and TGF-β1$^{223/225}$ gene transfer to the lung. Rats infected with control virus appeared healthy, and microscopic examination of the lung revealed a few neutrophils and mononuclear cells in the perivascular and peribronchial areas at days 1, 3, and 7 (Fig. 3 *a*) that did not extend into the parenchyma beyond, and that cleared rapidly, with no evidence of fibrosis (Fig. 3 *b*). Rats receiving AdTGF-β1 also appeared healthy, but showed a substantial mononuclear cell accumulation beginning in the perivascular and peribronchial areas and extending peripherally into the interstitium. This accumulation was absent at day 1, evident from day 3, peaked at day 7 (Fig. 3 *c*), and had largely resolved by day 14 (Fig. 3 *d*), in keeping with the kinetics of transgene protein expression. There was little evidence of fibroblast proliferation, or excess ECM protein deposition using either histochemical or immunohistochemical staining techniques (data not shown). In contrast, rats receiving AdTGF-β1$^{223/225}$ appeared lethargic, exhibited ruffled fur, and lost up to 33% of their body weight. One animal died on day 6. Macroscopically,

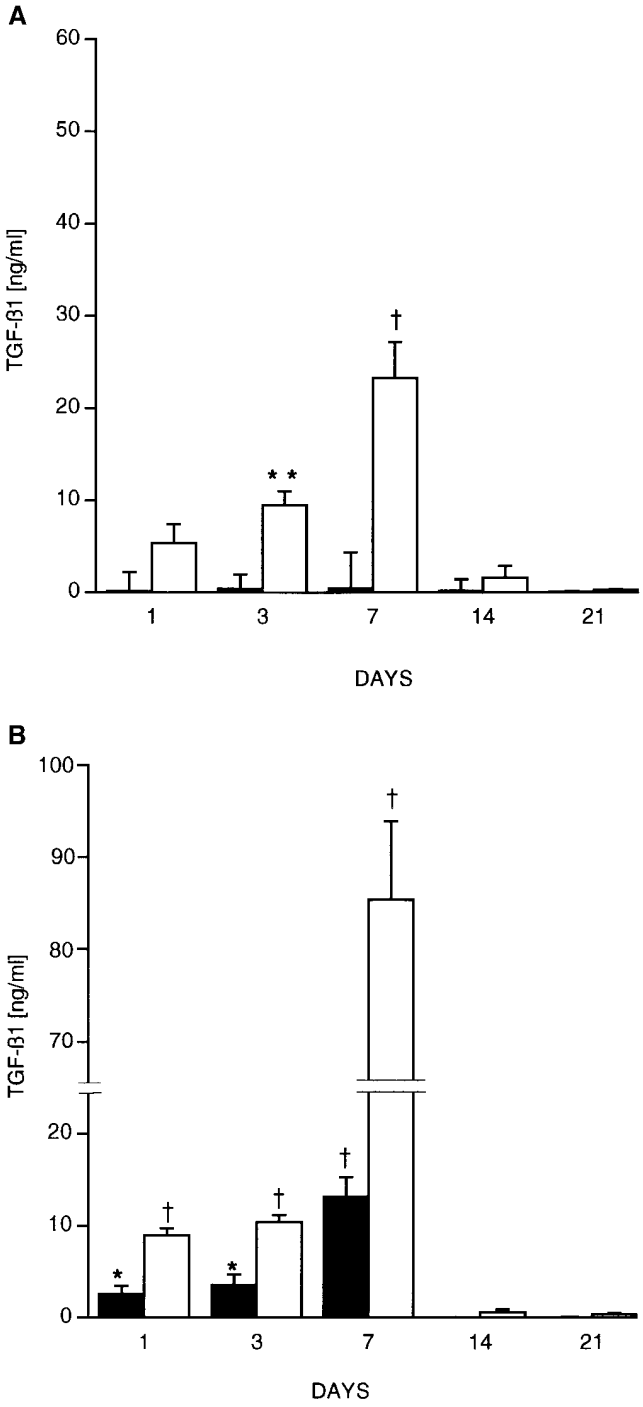


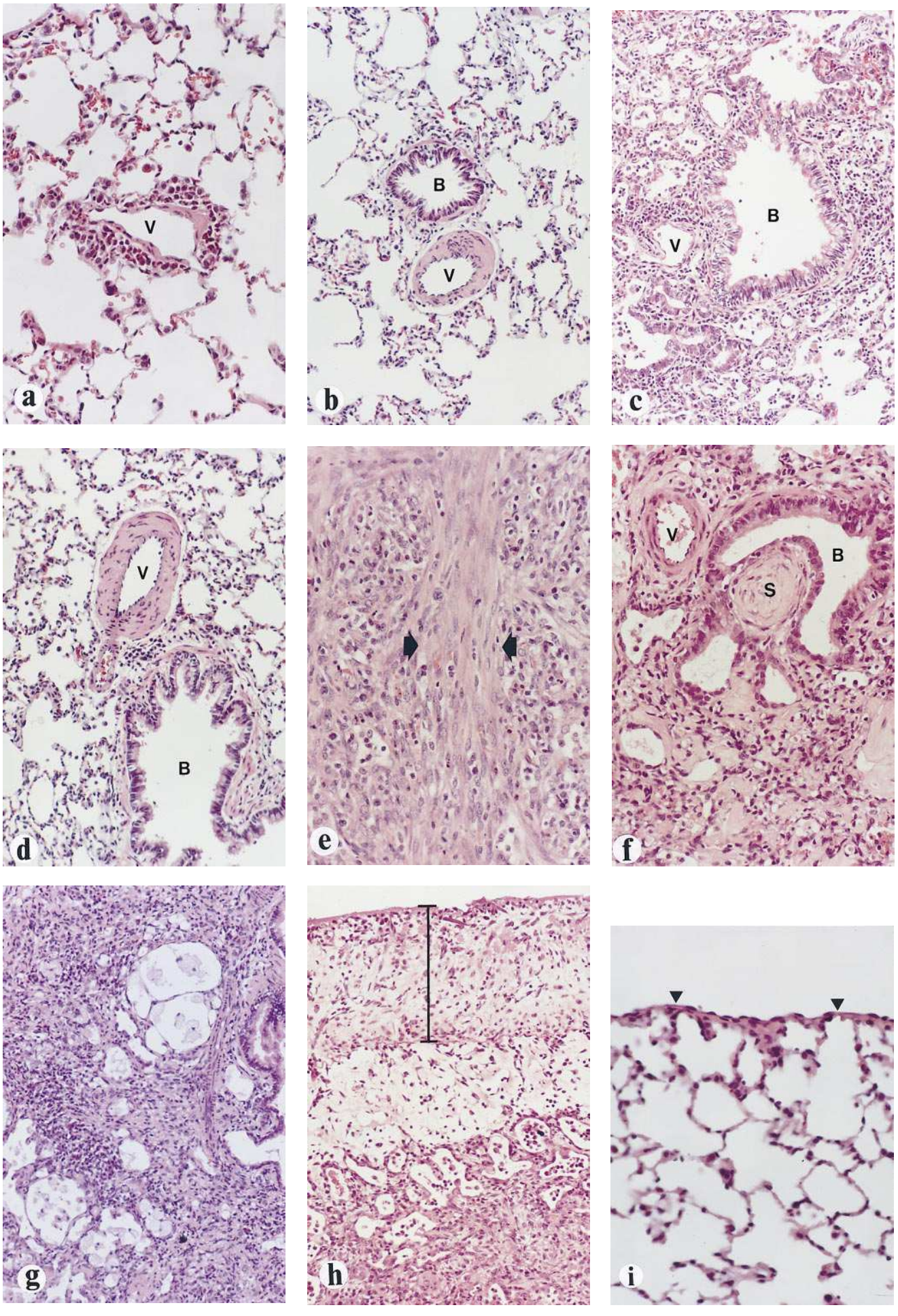
Figure 2. Levels of active and total (active + latent) TGF-β1 in the BAL of rats infected IT with the AdTGF-β1 (*A*) or AdTGF-β1$^{223/225}$ (*B*) and control (AdDL70-3) constructs. BAL samples were collected at various time points after infection, and were assayed for TGF-β1 protein by ELISA. Control virus resulted in production of < 300 pg/ml of latent protein. Results are expressed as mean±SEM from three to five animals/time point. *, **, and † indicate $P < 0.05$, $P < 0.005$, and $P < 0.0005$, respectively, between AdTGF-β1 (*A*) or AdTGF-β1$^{223/225}$ (*B*) and control virus. *Black bars*, active TGF-β1; *white bars*, total TGF-β1.

the lungs were markedly abnormal with areas of consolidation evident from day 7, with shrinkage and pleural adhesion formation evident from day 14 to day 64. Microscopically, at day 1 the lungs showed some perivascular and peribronchial edema, but no evidence of inflammatory cell or fibroblast accumulation. By day 3 there was patchy accumulation of mononuclear cells in peribronchial and perivascular areas, and to a lesser degree, in the adjacent alveolar spaces. Only scanty neutrophils (no eosinophils) were noted. There were areas of fibroblast accumulation around bronchi, and thickening of adjacent alveolar septae. By day 7, the inflammatory cell component was less marked, although there were still areas of mononuclear cell accumulation in peribronchial areas. Additionally, some eosinophils were noted, mainly around bronchi and vessels. Fibroblast accumulation was much more extensive, and now evident throughout the parenchyma (Fig. 3 *e*). These changes progressed, and by day 14 there was production of extensive fibrotic tissue with invasion into bronchial and alveolar structures, resulting in extensive destruction of normal lung architecture in some areas of the lung (Fig. 3 *f*). The changes were widespread, although somewhat patchy, probably due to the distribution of the virus after IT instillation. The inflammatory cell accumulation had largely resolved by this time point. The fibrotic changes continued to evolve, and persisted up to the last time point on day 64 (Fig. 3 *g*). In addition to these interstitial changes, the pleural surface was also extensively thickened from day 7 onwards. Fig. 3 *h* demonstrates the extensive pleural fibrosis seen at day 14 (Fig. 3 *h*), compared to pleural from a control rat (Fig. 3 *i*). The appearances of the large airways and pulmonary vessels were normal.

Fibroblast proliferation in both the pleura and interstitium was accompanied by evidence of extensive deposition of the ECM proteins collagen, elastin, and fibronectin from day 3 onwards. Collagen and elastin were detected by EVG staining, and Fig. 4, *a* and *b*, shows the extent of collagen deposition in scarred interstitium and pleura by day 14. Elastin expression was also increased, and was most evident at late time points (Fig. 4 *c*). Fibronectin immunostaining was intense at all time points in both the fibrotic interstitium and pleura (Fig. 4, *d* and *e*). Sections treated with the control antibody showed no immunostaining.

To characterize further the accumulation of fibroblasts and fibroblast-like cells in animals undergoing progressive fibrosis after AdTGF-β1$^{223/225}$ delivery, we examined tissues for induction of cells with the myofibroblast phenotype. The myofibroblast is a fibroblast-like cell characterized by the expression of αSMA (22), and thought to be important in fibrogenesis and wound healing (23). Using a monoclonal antibody against αSMA, we found immunopositivity in the vascular and bronchial smooth muscle layers in lungs treated with AdTGF-β1$^{223/225}$, as expected in normal lung. From day 3 onwards, however, there was also evidence of induction of αSMA expression in cells within fibroblastic areas, and by days 7 and 14 there was extensive and intense immunostaining within fibrotic areas, both in the interstitium (Fig. 4 *f*) and pleura. These αSMA-positive fibroblast-like cells also immunostained with the antivimentin antibody, making it likely that these cells were indeed myofibroblasts and not smooth muscle cells (data not shown). Staining in fibrotic areas persisted up to day 64. Sections treated with the control antibody showed no immunostaining.

No macroscopic or microscopic abnormalities were seen in any of the other major organs.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Quantitative measurement of collagen content after TGF-β1$^{223/225}$ gene transfer to the lung. Lungs were analyzed for their hydroxyproline content, and were compared to lungs from animals infected with control virus. There was an increase in mean hydroxyproline in rats treated with AdTGF-β1$^{223/225}$ compared to rats treated with control virus from day 14. This was statistically significant from day 21 onwards ($P < 0.01$) (Fig. 5), in keeping with the histopathological findings of extensive collagen deposition at these time points. By day 64 the hydroxyproline content increased by 95%. No such change was seen after overexpression of latent TGF-β1 (data not shown).

Discussion

To investigate the roles in vivo of TGF-β1, we have engineered two replication-deficient adenoviruses expressing latent and active forms of TGF-β1. Using similar adenoviruses expressing either the marker gene β-galactosidase or the cytokine interleukin 6 (IL-6) we have previously demonstrated that IT administration results in infection and transgene mRNA and protein expression in the epithelial cells of small and respiratory bronchioles as well as adjacent alveoli (13). In the present study after similar IT administration of recombinant adenoviruses, we have found that transient overexpression of the mutated (activated) transgene in pulmonary tissue results in extensive and persistent fibrosis, whereas transient overexpression of the native (latent) transgene induces only mononuclear infiltration. This demonstrates the importance of TGF-β1 in the pathogenesis of pulmonary fibrosis, and provides a new model of fibrosis to study aspects of fibrogenesis and the activation mechanism of TGF-β1 in the lung as well as other tissues.

One powerful method of investigating the role of TGF-β1 in pulmonary pathologies in vivo is to overexpress locally both active and latent TGF-β1 protein in a tissue-specific manner. There are different strategies to achieve this goal. Delivery of recombinant cytokines is limited by the short half-life of such cytokines, and the difficulties of repeated administration. The development of permanent transgenic animal models has overcome some of these problems, and has provided useful insights into the roles of a number of cytokines. For example, tissue-specific overexpression of TGF-β1 in pancreas (24) and liver (25) results in fibrosis. In these permanent transgenic models, however, the transgene is expressed throughout the lifetime (including development) of the animal, and may result in abnormal lung development (26), and as such may not accurately reflect the type of expression seen in human disease, where fibrosis evolves on the background of a normal mature lung, and cytokine expression is likely to be of shorter duration. We therefore chose a transient transgene approach using a replication-deficient recombinant adenovirus to overexpress latent or active TGF-β1 protein in the lung, and so assess the in vivo role(s) of the different forms of this cytokine. We have demonstrated that successful delivery of the transgene results in temporary transgene expression with peak BAL protein levels at 7 d, and a rapid decline by 14 d. Overexpression of the active and latent protein was compartmentalized with no apparent spill into the systemic circulation (data not shown). Delivery of the active AdTGF-β1$^{223/225}$ construct resulted not only in production of spontaneously active protein, but also high levels of latent protein (72.2 ng/ml at day 7). Previous studies of this mutated cDNA indicate that the TGF-β1 produced is largely spontaneously active (16). The origin of this latent protein is therefore uncertain at this time, but it is conceivable that it is derived from the rat itself by a process of autocrine stimulation of the endogenous rat TGF-β1 gene by active transgenic TGF-β1 protein. Such autoinduction has previously been demonstrated for TGF-β1 in vitro (2). Unfortunately, the high degree of homology between porcine and rat TGF-β1 precludes determination of the origin of the TGF-β1 protein using currently available reagents.

The histopathologic effects produced by overexpression of active TGF-β1 were dramatic. Inflammatory cell accumulation, predominantly mononuclear in nature, was evident from day 3. This was accompanied by the rapid onset of pulmonary fibrosis beginning in the perivascular and peribronchial areas, but quickly becoming widespread throughout the interstitium, extending up to and including the pleural surface, either as a result of diffusion of the cytokine itself, or autoinduction and activation of TGF-β1 in adjacent cells in a paracrine manner. These effects on fibroblast proliferation and stimulation of deposition of a variety of ECM proteins including collagen, elastin, and fibronectin are in accordance with the in vitro effects of TGF-β1 on fibroblasts (1), and the rapidity and severity of the fibrotic reaction also indicate that active TGF-β1 is an extremely potent fibrogenic cytokine in vivo. The emergence of αSMA-expressing fibroblast-like cells also suggests that overexpression of TGF-β1 in the lung has resulted directly or indirectly through induction of other mediators in development of a new cellular phenotype, the myofibroblast. Myofibroblasts have been demonstrated in a variety of fibrotic and healing tissues, and they are likely important in repair and fibrogenesis (23, 27). In the bleomycin model of fibrosis, most cells expressing procollagen mRNA have recently been demonstrated to be myofibroblasts (28). The origin of these myofibroblasts is uncertain, although currently the fibroblast is the most favored cellular precursor. While TGF-β1 has been clearly shown to induce the phenotype from fibroblasts in vitro and in skin in vivo (29), our data are the first to demonstrate directly that TGF-β1 is also a potent stimulus for generation of this phenotype in the lung. Since transgene expression was not significant after day 14, the fibrogenic process initiated by TGF-β1 overexpression had become self-perpetuating. It is possible that although active TGF-β1 was no longer detected in the BAL, it may still be active in local microenvironments within the lung

Figure 3. Histopathological effects induced by IT delivery of viruses AdDL70-3 (control), AdTGF-β1 (latent), and AdTGF-β1$^{223/225}$ (active). Sections are all stained with hematoxylin and eosin. *a* and *b* show days 7 and 14 after control virus (AdDL70-3) infection. *c* and *d* show days 7 and 14 after infection with AdTGF-β1 (latent). *e*, *f*, and *g* show days 7, 14, and 64 after infection with AdTGF-β1$^{223/225}$ (active). The arrows show fibroblastic response in interstitium. *S* is a mature scar, and *h* shows pleural changes day 14 after infection with AdTGF-β1$^{223/225}$. The bar shows thickened fibrotic pleura. (*i*) Pleura (*arrowhead*) of a control virus–infected rat at day 14. (*a–d* and *g*, ×250; *e*, *f*, *h*, and *i*, ×350. *B*, Bronchial structure. *V*, Vascular structure.

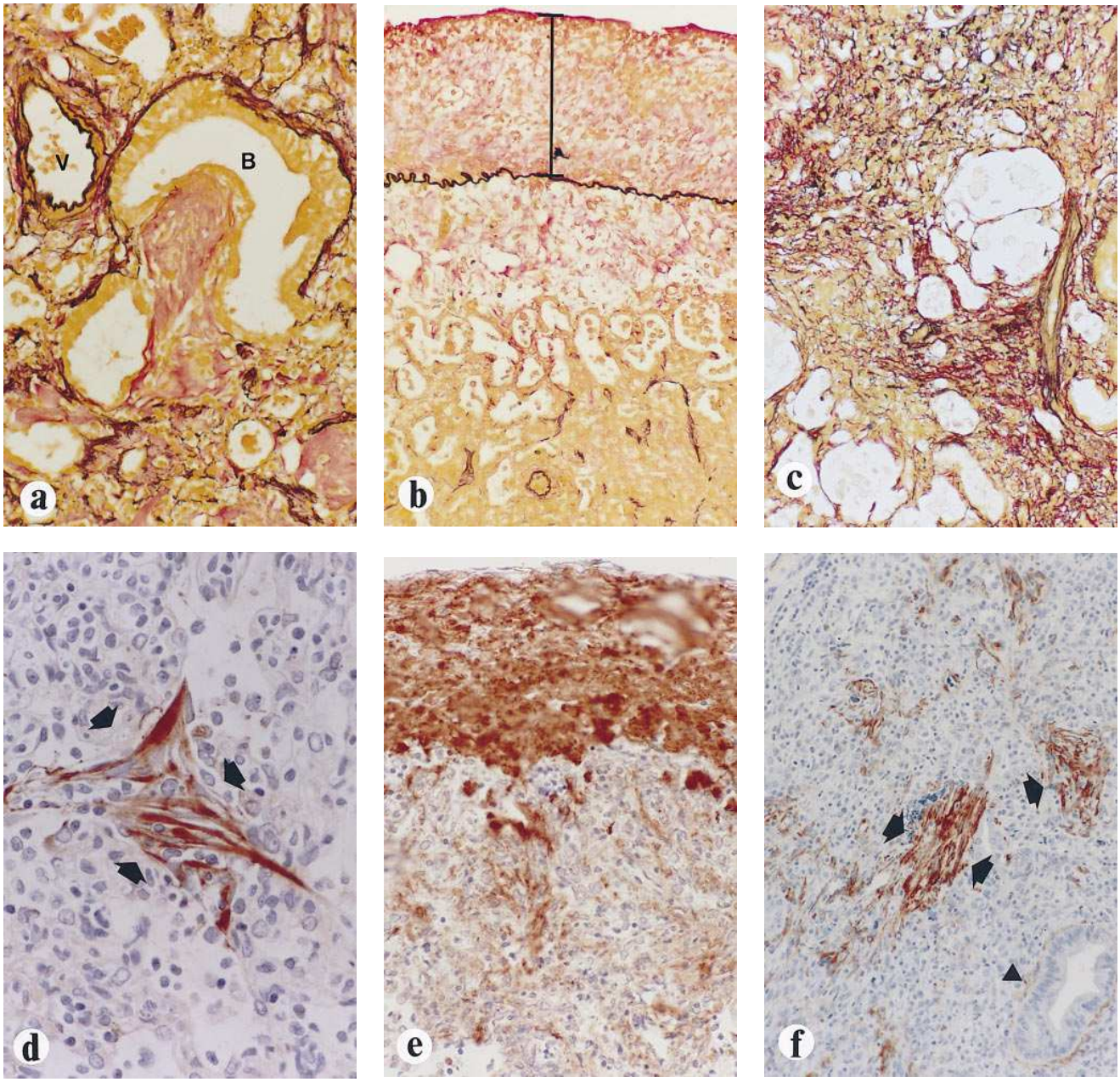

Figure 4. ECM deposition and induction of αSMA-positive myofibroblasts in rats after IT delivery of AdTGF-β1$^{223/225}$ (active). (*a–c*) Collagen red and Elastin black EVG staining. *a* and *b* are day 14 interstitium and pleura. *B*, bronchial structure; *V*, vascular structure; *bar*, fibrotic pleura; and *c* day 64 interstitium. *d* and *e*; Immunohistochemical localization of fibronectin in the interstitium (*arrows*) and pleura at day 14. (*f*) Immunohistochemical localization of αSMA on day 14. Immunopositive myofibroblasts, *arrows*; immunopositive bronchial smooth muscle, *arrowhead.* *a*, *b*, *d*, and *e*, ×350; *c* and *f*, ×250.

interstitium bound to components of the ECM such as the small proteoglycan biglycan (30), or fibronectin (31). In addition, TGF-β1 may not be the only fibrogenic cytokine active in this model, as TGF-β1 itself can stimulate macrophages to produce a variety of other fibrogenic cytokines, including TNF-α, platelet-derived growth factor, and basic fibroblast growth factor (1). Alternatively, the magnitude of the fibrotic process may be so great that it has overwhelmed the capacity of matrix-degrading enzymes to restore normal tissue architecture. Comparing the histological features of our model to those in the bleomycin model, the intensity of the fibrotic reaction induced by adenoviral overexpression of active TGF-β1 is more rapidly achieved and more severe, with increases in hydroxyproline at the higher end of those reported in various bleomycin studies (32). There is also less evidence of acute injury (particularly to the epithelium) and accompanying inflammation than in the bleomycin model.

In contrast to the effects of infection with AdTGF-β1$^{223/225}$, infection with latent AdTGF-β1 did not result in fibrosis, despite production of large quantities of latent TGF-β1 protein. This fact is likely to be the result of failure to activate this protein in vivo, and highlights the immense importance of TGF-β1

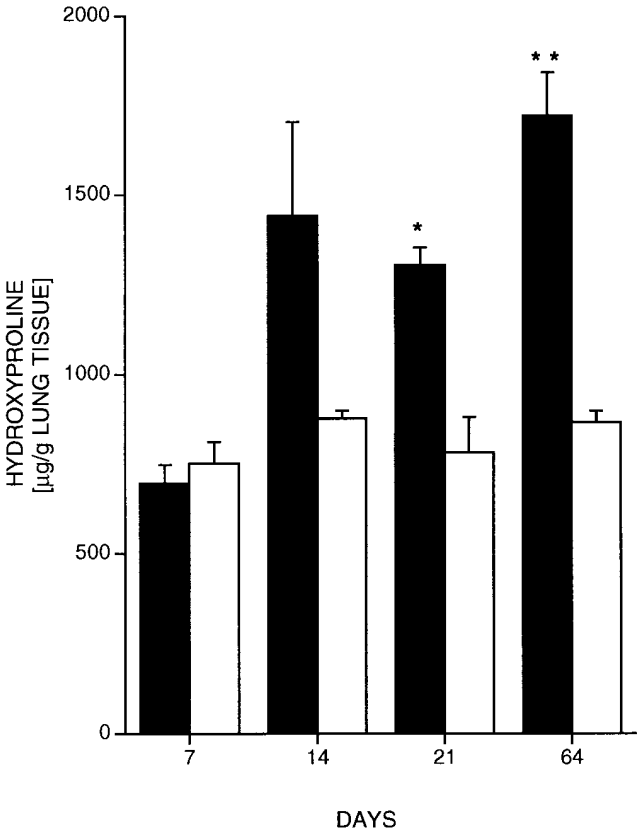

Figure 5. Hydroxyproline content of lung tissue was assayed after IT infection of rats with the AdTGF-β1$^{223/225}$ construct or control virus, and was expressed as the hydroxyproline content per wet weight lung tissue. Results are the means±SEM of three to five animals/time point. * and ** indicate $P < 0.01$, and $P < 0.005$, respectively, between AdTGF-β1$^{223/225}$ and control virus–infected animals. *Black bars*, hydroxyproline content after infection with AdTCF-β1$^{223/225}$; *white bars*, hydroxyproline content after control virus infection.

activation. The mononuclear infiltration that was observed may result from the small amounts of active cytokine produced, as the chemotactic effects of TGF-β1 in vitro on monocytes and macrophages can be seen at femtomolar concentrations (33). TGF-β1 gene expression alone is therefore unlikely to result in fibrosis unless there is activation of latent TGF-β1 protein by agents such as thrombospondin and the proteases plasmin and cathepsin (7, 8). This tight control of TGF-β1 activation is important as virtually all cells express the TGF-β receptors (34). When studying the expression of TGF-β1 in tissues, it is therefore important to differentiate the active from latent protein.

In terms of producing bioactive TGF-β1, the cellular source of the cytokine may be critical in fibrogenesis. Macrophages (35) and possibly lymphocytes (36) (at least in vitro) are capable of producing intrinsically active TGF-β1, which is in contrast to most other cell types. TGF-β1–producing macrophages are a prominent feature in a variety of fibrotic pulmonary pathologies (37). Khalil et al. (38) have recently demonstrated that alveolar macrophages isolated from animals with bleomycin-induced fibrosis are capable of producing significant quantities of both active and latent TGF-β1 protein, and the time course of production of active cytokine parallels secretion of plasmin by these macrophages. Whether the macrophage-derived plasmin can also activate latent protein from other cells is not known. Targeting this macrophage-derived bioactive TGF-β1 protein may become a valuable antifibrotic strategy.

In conclusion, overexpression of active, but not latent, TGF-β1 results in production of rapid and severe fibrosis, and this highlights the need for future study of the in vivo activation mechanisms of this cytokine. Moreover, we have developed a new model of fibrosis that can be used for studying the pathogenetic mechanisms of and therapeutic interventions in pulmonary fibrosis.

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