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Transforming Growth Factors-β_1, -β_2, and -β_3 Stimulate Fibroblast Procollagen Production *in Vitro* but Are Differentially Expressed during Bleomycin-Induced Lung Fibrosis

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Transforming growth factor (TGF)-β_1 may potentiate wound healing and fibrosis by stimulating fibroblast collagen deposition. TGF-β_1 is implicated in the pathogenesis of pulmonary fibrosis, but the role of TGF-β_2 and TGF-β_3 remains unclear. We examined their effects on lung fibroblast procollagen metabolism* in vitro *and localized their gene expression during bleomycin-induced lung fibrosis using* in situ *hybridization with digoxigenin-labeled riboprobes. All three isoforms stimulated fibroblast procollagen production. TGF-β_3 was the most potent and also reduced procollagen degradation. In normal mouse lung, TGF-β_1 and TGF-β_3 mRNA transcripts were abundant in bronchiolar epithelium. After bleomycin, TGF-β_1 gene expression was maximally enhanced at 10 days, with the signal being predominant in macrophages. Signal was also enhanced in mesenchymal, pulmonary endothelial, and mesothelial cells. After 35 days, the pattern of TGF-β_1 gene expression returned to that of control lung. TGF-β_3 gene expression remained unchanged throughout compared with controls. TGF-β_2 mRNA was not detected with the antisense probe, but signal obtained with the sense probe suggests the presence of a naturally occurring antisense. This study demonstrates that TGF-β_1, -β_2, and -β_3 all exert profibrotic effects* in vitro. *However, TGF-β isoform gene expression is differentially controlled during experimental pulmonary fibrosis with TGF-β_1 the predominant isoform expressed during pathogenesis. (Am J Pathol 1997, 150:981–991)

The pathogenesis of pulmonary fibrosis remains incompletely understood. One current hypothesis is that initial endothelial or epithelial cell injury triggers an influx of inflammatory cells from the circulation. Cytokines derived from these inflammatory cells as well as from resident cells then stimulate fibroblasts to synthesize excessive amounts of extracellular matrix, including collagen.[1]

One such group of cytokines is the transforming growth factor (TGF)-β family. Three mammalian TGF-β isoforms are now recognized, TGF-β_1, -β_2, and -β_3. TGF-β_1 is an extremely potent promoter of extracellular matrix accumulation and acts *via* both transcriptional and post-transcriptional mechanisms.[2] The effects of TGF-β_2 and TGF-β_3 on fibroblast collagen synthesis and degradation have not been studied.

There is now considerable evidence implicating TGF-β_1 in the pathogenesis of pulmonary fibrosis. TGF-β_1 and its mRNA levels increase during the development of experimentally induced lung fibrosis,[3,4] and TGF-β_1 antibodies attenuate the fibrotic response in the bleomycin mouse model[5] and in immune-induced lung fibrosis.[6] TGF-β_1 protein synthesis is increased in patients with idiopathic pulmo-

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nary fibrosis[7,8] and with other interstitial lung diseases.[9]

Differential TGF-β isoform expression during development,[10–12] in normal adult tissues,[13–15] and in disease[16,17] suggests that TGF-β_{1-3} may each have different functions *in vivo*. However, the role of TGF-β_2 and TGF-β_3 in the pathogenesis of pulmonary fibrosis is at present controversial. Three studies of bleomycin-induced pulmonary fibrosis suggest that TGF-β_1 is the predominant isoform involved,[18–20] whereas another proposes that all three isoforms may be implicated.[21]

The aim of this study was to examine the role of TGF-β_2 and TGF-β_3 in the pathogenesis of pulmonary fibrosis. We initially investigated whether TGF-β_2 and TGF-β_3 shared the ability of TGF-β_1 to promote extracellular matrix accumulation *in vitro*. Dose-response studies showed that TGF-β_{1-3} all stimulate fibroblast procollagen synthesis. TGF-β_3 was the most potent isoform in this assay and also reduced intracellular procollagen degradation. Having established that all three isoforms are potentially profibrotic *in vitro*, we examined their gene expression by *in situ* hybridization in a murine model of lung fibrosis. Our results show that gene expression of the three isoforms is differentially regulated during the development of bleomycin-induced lung fibrosis. TGF-β_1 but not TGF-β_2 or TGF-β_3 gene expression was enhanced after bleomycin, suggesting that TGF-β_1 is the predominant isoform implicated in the pathogenesis of this disease.

Materials and Methods

Cell Culture

Procollagen production was determined using previously published methods.[2] Human fetal lung fibroblasts (HFL-1, American Type Culture Collection, Rockville, MD) were cultured in 12-well plates with Dulbecco's modified Eagle's medium plus 5% newborn calf serum in a humidified atmosphere containing 10% CO_2 at 37°C until confluent. Dulbecco's modified Eagle's medium was then removed and replaced with 1 ml of preincubation medium containing 4 mmol/L glutamine, 50 μg/ml ascorbic acid, 0.2 mmol/L proline, and 2% newborn calf serum. After 24 hours, the preincubation medium was removed and replaced with 1 ml of fresh preincubation medium containing one of the TGF-β isoforms at concentrations ranging from 0.05 to 5 ng/ml (2 to 200 pmol/L). Control cells had medium replaced without the addition of TGF-β. TGF-β_1 and TGF-β_2 were natural porcine and TGF-β_3 was recombinant chicken (R&D Systems, Abingdon, UK). Cells were then incubated for an additional 24 hours before harvesting. Collagen production has previously been shown to be linear over this period of time.[22]

The cell layer was scraped into the medium and aspirated. Each well was washed with 1 ml of phosphate-buffered saline (PBS) and the washings combined with the initial aspirate. Ethanol was added to a final concentration of 67% (v/v) and proteins precipitated at 4°C overnight. The samples were then filtered (0.45 μm) as described previously.[23] After hydrolysis in 2 ml of 6 mol/L hydrochloric acid overnight at 110°C and subsequent charcoal filtration (0.65 μm), hydroxyproline was derivatized with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole and separated using high pressure liquid chromatography.[24] Hydroxyproline content was then determined by comparison with standards containing known amounts of hydroxyproline and derivatized under the same conditions. The linearity of the hydroxyproline assay has previously been established between 5 pmol and 20 nmol.[24] Procollagen production was calculated from the quantity of hydroxyproline present in the ethanol-insoluble fraction. The proportion of newly synthesized procollagen degraded intracellularly (expressed as a percentage of total procollagen synthesis) was calculated from the quantity of hydroxyproline in the ethanol-soluble fraction compared with that in both the ethanol-soluble and insoluble fractions.[2] Total procollagen synthesis is represented by the sum of hydroxyproline in the ethanol-soluble and -insoluble fractions.

Animals

Adult mice (strain $B_6D_2F_1$) aged 8 weeks and weighing 24 to 26 g received a single dose of intratracheal saline (0.14 mol/L) or saline containing bleomycin sulfate (6 mg/kg) in a volume of 0.05 ml and were killed 3, 10, 21, or 35 days later by pentobarbitone overdose as previously described.[25] Nine animals were used in each group: six for hydroxyproline estimations and three for *in situ* hybridization studies. Lungs were fixed by intratracheal instillation of freshly prepared 4% paraformaldehyde in PBS at a pressure of 25 cm H_2O. The trachea was ligated just caudal to the larynx and the thoracic contents removed together. After 4 hours of immersion in fixative, lung tissue was transferred to 15% sucrose in PBS before dehydration and embedding in paraffin wax.

For total lung collagen estimation, tissue hydroxyproline content was measured as previously described.[26] Briefly, this was determined spectro-

photometrically after oxidation with chloramine T and extraction of the toluene-miscible product. Collagen content was calculated from hydroxyproline content assuming that lung collagen contains 12.2% w/w hydroxyproline.[27]

Tissue Preparation

Two sections were cut from each block and stained for collagen with Massons' trichrome. Prehybridization treatments were performed using techniques described previously.[28,29] Sections (5 μm thick) were cut and placed on slides previously coated with a 2% v/v solution of 3-aminopropyltriethoxysilane in acetone. After dewaxing, sections were rehydrated through a series of ethanol washes of decreasing concentration, followed by immersion in 0.14 mol/L sodium chloride and PBS before refixing in freshly prepared 4% paraformaldehyde. To maximize entry of the probe into cells, sections were treated with proteinase K (Life Technologies, Paisley, UK) at a concentration of 20 μg/ml in 50 mmol/L Tris hydrochloride, pH 7.5, 5 mmol/L EDTA for 10 minutes before refixing with paraformaldehyde. Sections were then acetylated by immersion in freshly prepared 0.1 mol/L triethanolamine containing 0.25% acetic anhydride and subsequently dehydrated through a series of increasing concentrations of ethanol.

Probe Preparation

Riboprobes were synthesized from transcript-specific murine TGF-β cDNA constructs in pGEM vectors (TGF-β_1 and TGF-β_3) and in the SP72 vector (TGF-β_2). The constructs were obtained by deleting the highly conserved regions of the three murine cDNAs, and the specificity of riboprobes synthesized from these templates is established.[17,30] The TGF-β_1 construct includes nucleotides 421 through 1395 of the murine DNA and contains 764 bp of the amino-terminal glycopeptide (precursor) region and 210 bp of the mature region. The TGF-β_2 construct contains 442 bp of the amino-terminal glycopeptide region (1511 through 1953). The TGF-β_3 construct contains 609 bp of the amino-terminal glycopeptide region (831 through 1440). Digoxigenin-labeled riboprobes were synthesized by *in vitro* transcription from the linearized cDNA templates using the appropriate RNA polymerase (SP6 or T7) according to the manufacturer's protocol (Boehringer Mannheim, Lewes, UK). For Northern analysis, cDNA probes were labeled with ^{32}P to a specific activity of 2×10^6 dpm/ng by random prime labeling (Amersham, Slough, UK) according to the manufacturer's instructions.

Northern Analysis

Northern analysis was performed to confirm probe specificity. Total RNA was extracted from murine lung using previously described methods.[28,29] Poly A^+ RNA was selected with a biotinylated oligo (dT) probe (Promega, Southampton, UK). Hybrids were then captured and washed at high stringency using streptavidin coupled to paramagnetic particles and a magnetic separation stand (Promega). Poly A^+ RNA was subsequently eluted with water. The 10-μg samples of poly A^+ RNA were electrophoresed through an agarose-formaldehyde gel and blotted onto a Hybond N nylon membrane (Amersham). Hybridizations were performed overnight at 42°C in 50% formamide, 5X saline sodium phosphate, 5X Denhardt's reagent, 0.1% sodium dodecyl sulfate (SDS), and 100 μg/ml denatured salmon sperm DNA. Post-hybridization washes were performed in 2X standard saline citrate (SSC), 0.1% SDS for 10 minutes at 42°C twice followed by 1X SSC, 0.1% SDS for 15 minutes at 42°C. Hybridized probe was detected by autoradiography followed by scanning with laser densitometry.

In Situ *Hybridization*

This protocol was based on previously described methods.[25] Hybridization buffer consisting of 50% deionized formamide, 300 mmol/L NaCl, 20 mmol/L Tris/HCl (pH 7.4), 5 mmol/L EDTA, 10 mmol/L monosodium phosphate (pH 8.0), 10% dextran sulfate, 1X Denhardt's solution, and 500 μg/ml yeast tRNA was mixed with digoxigenin-labeled probe at a ratio of 9:1 to give a final probe concentration of 20 ng/ml. A 25-μl aliquot of hybridization solution was applied to each slide, and sections were incubated overnight (16 hours) at 50°C in a sealed chamber humidified with a solution of 50% formamide in 2X SSC. After hybridization, all incubations were performed at room temperature. Slides were washed in 4X SSC for 30 minutes and then in 0.2X SSC for 30 minutes. Slides were then washed in Tris-buffered saline (TBS, consisting of 0.1 mol/L Tris, pH 8.2, 0.15 mol/L NaCl) for 5 minutes. They were then incubated for 30 minutes with antibody blocking solution consisting of 5% bovine serum albumin and 5% normal sheep serum diluted in TBS with 0.1% Tween 20 (Sigma, Dorset, UK). After two additional 5-minute washes in TBS, the slides were incubated with antibody solution for 30 minutes. This consisted of a

1:100 dilution of anti-digoxigenin-alkaline phosphatase Fab fragments (Boehringer Mannheim) in 1% bovine serum albumin in TBS with Tween 20. Sections were then washed twice in TBS for five minutes each.

For detection of bound antibody, sections were incubated with alkaline phosphatase substrate New Fuschin Red (Dako, High Wycombe, UK). This was prepared according to the manufacturer's instructions, and 1 mmol/L levamisole was added to inhibit endogenous alkaline phosphatase activity.[31] A 200-μl aliquot of freshly prepared reagent was applied to each section for 20 minutes. The slides were then rinsed in distilled water and counterstained with hematoxylin or methyl green. They were subsequently mounted in glycerol without dehydration. The New Fuschin Red yields a red color at the site of the hybridized probe. Each *in situ* experiment was repeated at least twice at each time point. Sections were examined and reported independently by three of the authors (R. K. Coker, P. K. Jeffery, and R. J. McAnulty).

Statistical Analysis

Data were analyzed with an unpaired *t*-test or one-way analysis of variance for multiple comparisons using the Newman-Keuls procedure. Mean values for collagen production, degradation, and synthesis were held to be significantly different when the the probability of such differences arising, assuming the null hypothesis to be true, were less than 5% ($P < 0.05$). Where mean values are calculated, standard errors of the mean (SEM) are also given.

Results

In Vitro *Studies*

Dose-response curves obtained for each of the three TGF-β isoforms are shown in Figure 1. Lung fibroblast procollagen production is shown as a function of TGF-β concentration in the culture medium during the 24-hour incubation period. Data are the means ± SEM of four to six replicate cultures per condition. The results demonstrate that all three isoforms stimulated fibroblast procollagen production in a dose-dependent manner. TGF-β_3 was the most potent isoform, exerting a maximal effect at a concentration of 4 pmol/L, whereas the other two peptides attained a maximal stimulation only between 20 and 40 pmol/L. Concentrations above 40 pmol/L did not further stimulate procollagen production. Although TGF-β_3 achieved maximal stimulation at a lower concentration than TGF-β_1 or TGF-β_2, the maximal response was similar, with increases of approximately 50% above control values for all three isoforms.

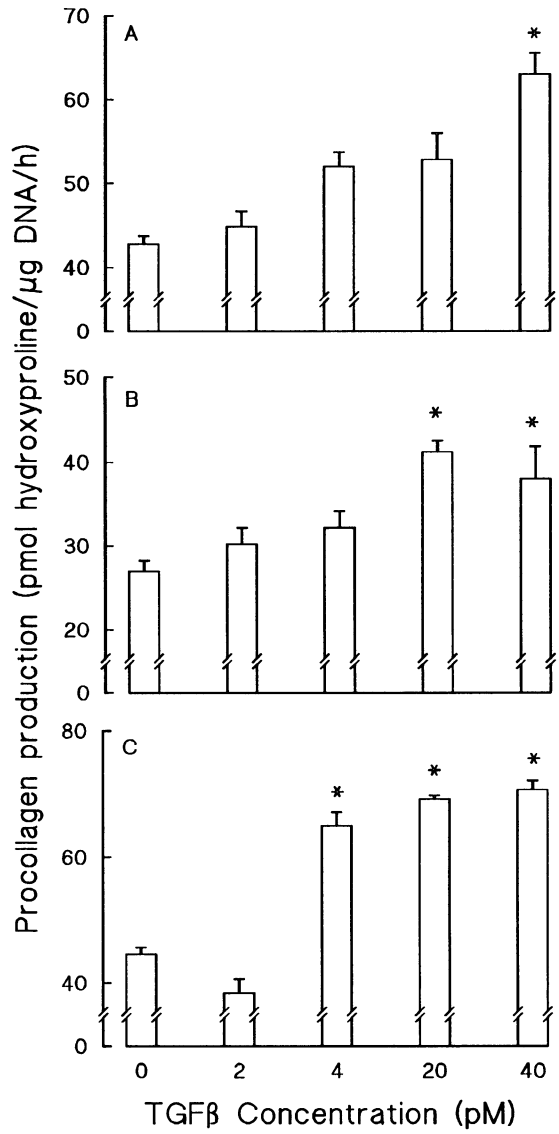

Figure 1. *The effect of TGF-β isoforms on lung fibroblast procollagen production. HFL-1 procollagen production, assessed by measurement of hydroxyproline, is shown as a function of TGF-β isoform concentration. Each value represents the mean ± SEM from four to six replicate cultures.* A: *TGF-β_1*. B: *TGF-β_2*. C: *TGF-β_3*. *P < *0.01 compared with control.*

In this set of studies, the basal rate of procollagen production for experiments with TGF-β_2 was lower than that for TGF-β_1 and TGF-β_3. The basal rate varied between experiments but was always between 27 and 45 pmol/μg DNA/hour.

Figure 2 shows the effects of each of the TGF-β isoforms on lung fibroblast procollagen production, procollagen synthesis, and intracellular degradation at a concentration of 40 pmol/L, which produced

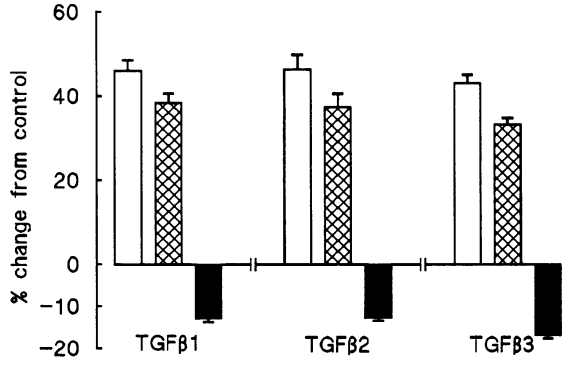

Figure 2. *The effect of TGF-β_1, -β_2, and -β_3 on lung fibroblast procollagen metabolism. Each value represents the mean percentage change from control ± SEM from four separate experiments, each containing four to six replicate cultures.* Open bars, *procollagen production;* hatched bars, *procollagen synthesis;* solid bars, *procollagen degradation. Procollagen production is calculated from the quantity of hydroxyproline present in the ethanol-insoluble fraction. Procollagen degradation (expressed as a percentage) is calculated from the amount of procollagen present in the ethanol-soluble fraction, whereas procollagen synthesis represents the sum of procollagen present in ethanol-insoluble and ethanol-soluble fractions.*

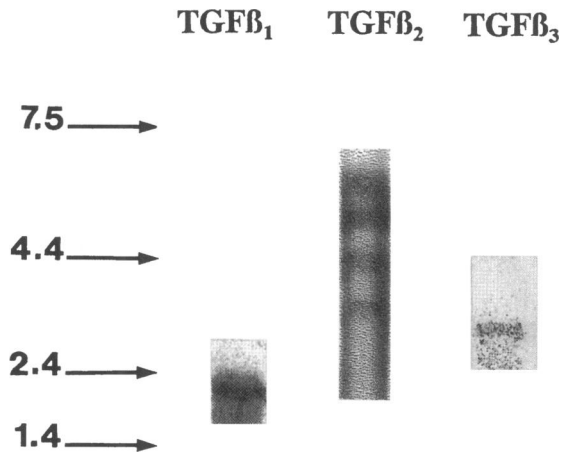

Figure 3. *Northern analysis of murine lung with TGF-β_{1-3} probes. Ten micrograms of poly A$^+$ RNA from murine lung was hybridized with each of the three radiolabeled cDNA probes. Size markers (in kilobases) are indicated on the left.*

maximal stimulation of procollagen production by all three isoforms. Data are the means ± SEM of four experiments, each consisting of four to six replicate cultures. TGF-β_1, TGF-β_2, and TGF-β_3 stimulated procollagen production by approximately 46%, 46%, and 43%, respectively ($P < 0.01$ in all cases). Procollagen synthesis was significantly increased by each of the three isoforms. Control synthesis rose by approximately 38%, 37%, and 33% with TGF-β_1, TGF-β_2, and TGF-β_3 respectively ($P < 0.01$ in all cases). There were no significant reproducible differences between the three isoforms in terms of their effects on either procollagen production or synthesis at this concentration. There was a tendency for all three isoforms to reduce intracellular procollagen degradation, but this was significant only in the case of TGF-β_3 ($P < 0.05$).

In Vivo *Studies*

Table 1 shows the time course of changes in lung collagen content during the development of bleomycin-induced pulmonary fibrosis. Total lung collagen increased with age and was slightly higher in control animals after 35 days. Total lung collagen in bleomycin-treated animals was unchanged at 3 days but 10 days after treatment had risen by approximately 40% ($P < 0.05$). It continued to rise and by 35 days values were approximately 80% above those for control animals ($P < 0.01$).

Staining of lung sections with Massons' trichrome revealed extensive fibrosis 35 days after bleomycin (data not shown). Taken together, the results of total lung collagen estimation and histological staining for lung collagen confirm that these mice developed lung fibrosis in response to bleomycin.

Figure 3 shows Northern analysis of poly A$^+$ RNA from murine lung. The following bands were seen: TGF-β_1, 2.2 kb; TGF-β_2, four bands between 3.5 and 6.3 kb; TGF-β_3, 3.3 kb. These transcripts are similar to those previously published[12] and these results therefore confirm the specificity of the probes.

Figure 4 shows lung tissue from a representative control animal hybridized with TGF-β_1 (a, antisense; b, sense), TGF-β_2 (c, antisense; d, sense), and TGF-β_3 (e, antisense; f, sense) probes. Both TGF-β_1 and TGF-β_3 mRNA transcripts, stained red, were

Table 1. *Time Course of Changes in Lung Collagen Content during the Development of Bleomycin-Induced Pulmonary FIbrosis*

	Lung collagen content (mg)			
	3 days	10 days	21 days	35 days
Control	1.24 ± 0.02	1.10 ± 0.01	1.23 ± 0.03	1.60 ± 0.02
Bleomycin	1.20 ± 0.04	1.61 ± 0.08*	1.76 ± 0.14†	2.89 ± 0.14†

Values are the mean (n = 6) ± SEM.
*$P < 0.05$ compared with controls.
†$P < 0.01$ compared with controls.

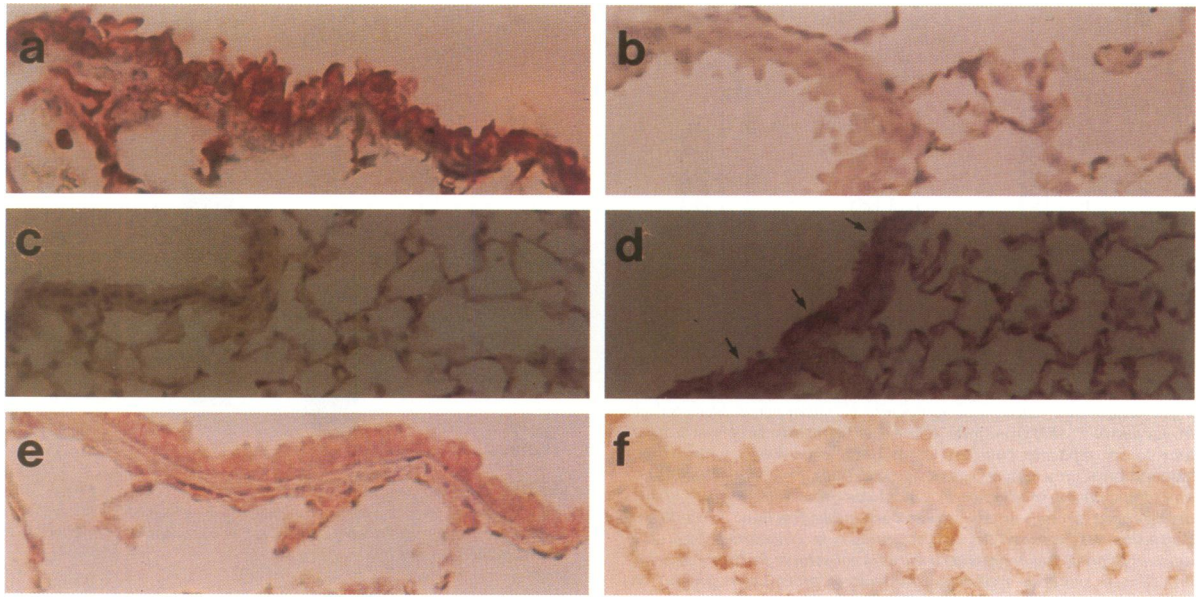

Figure 4. *TGF-β_{1-3} gene expression in normal murine lung.* **a** *and* **b**: *Normal murine lung hybridized with TGF-β_1 antisense* (**a**) *and sense* (**b**) *riboprobes.* **c** *and* **d**: *Normal murine lung hybridized with TGF-β_2 antisense* (**c**) *and sense* (**d**) *riboprobes.* **Arrows** *show hybridization signal obtained with the sense probe in bronchial epithelium* (**d**). **e** *and* **f**: *Normal murine lung hybridized with TGF-β_3 antisense* (**e**) *and sense* (**f**) *riboprobes. Hematoxylin counterstain* (**a** *to* **d**); *methyl green* (**e** *and* **f**); *original magnification, ×200. Hybridization signal appears as a red color at the site of mRNA detection.*

present in normal mouse lung, and gene expression for both isoforms was predominant in bronchiolar epithelial cells. However, mRNA transcripts for TGF-β_1 and TGF-β_3 were also observed in the interstitium, where they were localized to alveolar macrophages, mesenchymal cells, and cells lining the alveolar wall thought to be alveolar type II cells. Signal for both isoforms was also detectable in mesothelial cells at the pleural edge (not shown).

The TGF-β_2 probes generated hybridization signal with the sense probe (arrowed in bronchial epithelium in Figure 4d) but little or no signal with the antisense probe (Figure 4c). Cellular localization of signal obtained with TGF-β_2 sense probe was similar to, but less widespread, than that obtained with the TGF-β_1 antisense probe. These results were reproducible between experiments and between different batches of probe. Probe orientation was therefore independently confirmed using two methods. First, asymmetric restriction enzyme cuts were performed with *Cla*I and the resulting fragments examined by agarose gel electrophoresis. Second, dideoxy-mediated chain termination sequencing (United States Biochemical, Amersham, UK) yielded a sequence pattern that confirmed the greatest homology (87%) of the sense probe with mRNA for murine TGF-β_2[32,33] when matched using the FASTA database searching program (Human Genome Mapping Project CRC, Cambridge, UK). The possibility of greater digoxigenin labeling of the sense probe compared with the antisense probe was excluded using a chemiluminescence assay (Boehringer Mannheim) according to the manufacturer's instructions. The ratio of digoxigenin labeling of the antisense probe compared with the sense probe was 1.2 to 1.

TGF-β_1 gene expression was only slightly enhanced 3 days after bleomycin (not shown) and appeared maximally enhanced 10 days after bleomycin administration. Figure 5 shows lung tissue at this time hybridized with TGF-β_1 (a, sense; b–e, antisense), TGF-β_2 (f, sense; g, antisense), and TGF-β_3 (h, sense; i, antisense) probes. After bleomycin, reduced TGF-β_1 gene expression was apparent in bronchiolar epithelium (arrows in Figure 5b). Inflammation at this time was patchy and localized mainly around airways and blood vessels and beneath the pleura. TGF-β_1 gene expression was predominantly localized to inflammatory cells including macrophages (arrowheads in Figure 5c) but was also present in increased intensity and in greater numbers of mesenchymal cells underlying blood vessels (arrows in Figure 5c), luminal cells that may be endothelial or subendothelial (Figure 5d), and in mesothelial and submesothelial cells adjacent to areas of subpleural fibrosis (Figure 5e). In addition, there was intense signal throughout the interstitium, consistent with expression of TGF-β_1 by capillary endothelial cells, alveolar type I epithelial cells, and

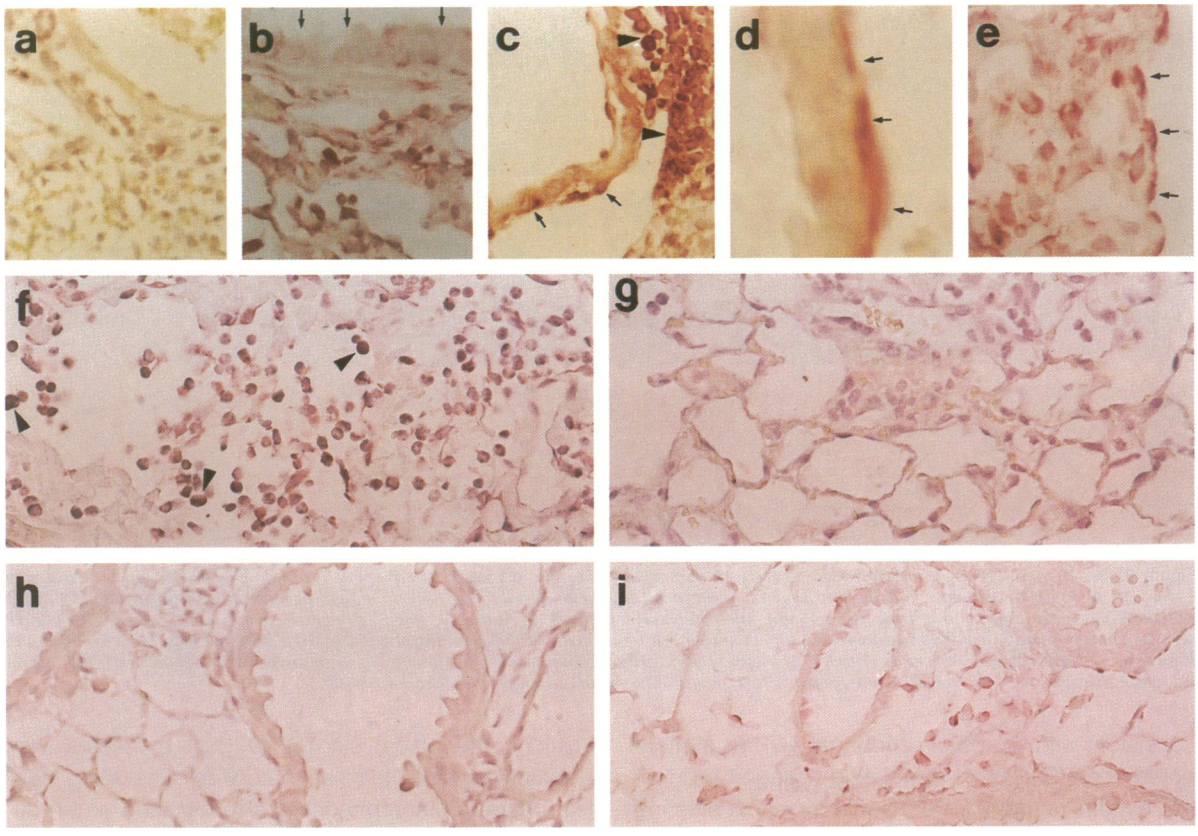

Figure 5. *TGF-β_{1-3} gene expression 10 days after bleomcyin.* a *to* e: *TGF-β_1 gene expression. Marked inflammatory cell infiltrate but absence of signal with sense probe* (a). *Hybridization signal was reduced in bronchiolar epithelium* (arrows *in* b) *but enhanced in alveolar macrophages and inflammatory cells* (arrowheads *in* c) *and mesenchymal cells underlying blood vessels* (arrows *in* c). *Signal was also increased in luminal cells, which may be pulmonary endothelial or subendothelial cells* (arrows *in* d), *and in mesothelial cells adjacent to areas of subpleural fibrosis* (arrows *in* e). f *and* g: *TGF-β_2 gene expression. Positive signal was obtained with sense probe* (arrowheads *in* f), *but little signal was observed with antisense probe* (g). h *and* i: *TGF-β_3 gene expression, showing absence of signal with sense probe* (h). *Very little signal was observed in bronchiolar epithelium, macrophages, or inflammatory foci. Hematoxylin counterstain; original magnification, ×200* (a, b, *and* f *to* i), *×400* (c *and* e), *and ×1000* (d).

fibroblasts. Cells lining alveolar walls, probably alveolar type II cells, also expressed TGF-β_1 (not shown).

Hybridization with the TGF-β_2 riboprobes again yielded positive signal with the sense probe (arrowheads in Figure 5f) but little or no signal with the antisense probe. In contrast to TGF-β_1, TGF-β_3 gene expression was not enhanced after bleomycin, and very little signal was observed either in bronchiolar epithelium or in macrophages and inflammatory foci.

Figure 6 shows lung tissue examined 21 and 35 days after bleomycin hybridized with TGF-β_1 sense (Figure 6, a and c) and antisense (Figure 6, b and d) probes, respectively, and lung tissue 21 days after bleomycin hybridized with the TGF-β_2 sense (Figure 6e) and antisense (Figure 6f) probes. Patchy fibrosis was evident at 21 days with TGF-β_1 expression predominant in alveolar macrophages (arrowheads in Figure 6b). At 35 days after bleomycin, there was a return toward the control pattern of gene expression for TGF-β_1, with mRNA transcripts predominant in bronchiolar epithelium (arrowheads in Figure 6d). Similarly, at 35 days, the pattern of signal localization for TGF-β_3 was identical to that seen in control animals (data not shown). Hybridization with the TGF-β_2 probes again yielded positive signal with the sense probe (arrowheads in Figure 6e) but little or no signal with the antisense probe.

Discussion

In this study we have examined the effects of TGF-β_{1-3} on fibroblast procollagen synthesis and degradation. We have shown that TGF-β_2 and TGF-β_3 share the ability of TGF-β_1 to promote collagen deposition by stimulating fibroblast procollagen synthesis. This is consistent with their high degree of homology and previous assays demonstrating similar biological effects.[34] However, TGF-β_3 was 10 times more potent than the other two isoforms in stimulating procollagen production and also reduced intracellular procollagen degradation. Our findings are

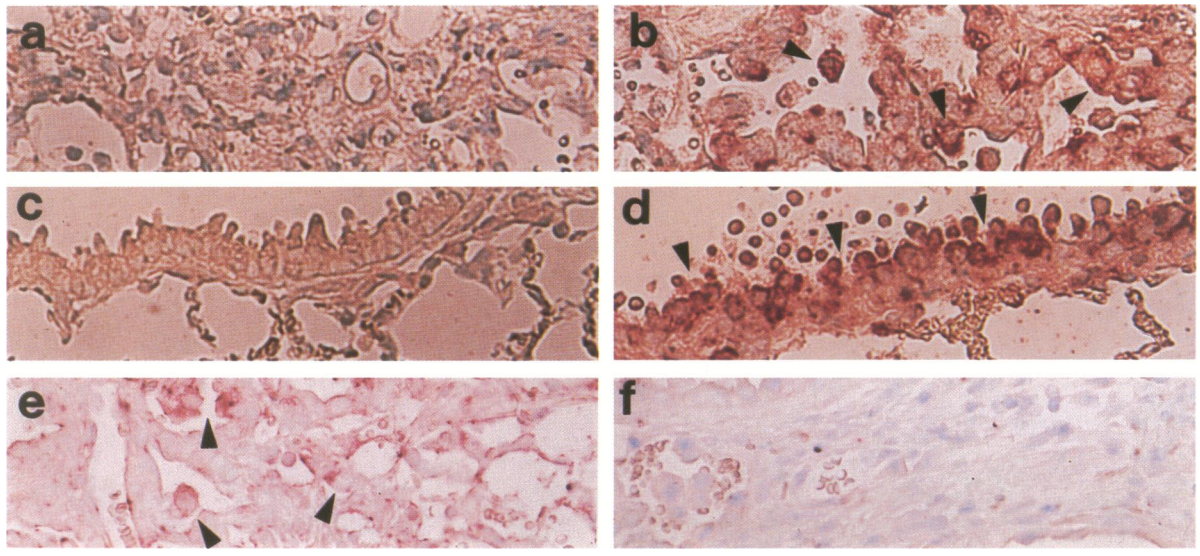

Figure 6. *TGF-β_1 and TGF-β_2 gene expression 21 and 35 days after bleomycin.* a *and* b: *TGF-β_1 gene expression in lung tissue 21 days after bleomycin.* a: *Sense.* b: *Antisense. Patchy fibrosis was evident at 21 days, with signal for TGF-β_1 prominent in macrophages* (arrowheads *in* b). c *and* d: *TGF-β_1 gene expression in lung tissue 35 days after bleomycin.* c: *Sense.* d: *Antisense. TGF-β_1 gene expression returned toward the pattern seen in control animals, with hybridization signal predominant in bronchiolar epithelium* (arrowheads *in* d). e *and* f: *TGF-β_2 gene expression in lung tissue 21 days after bleomycin.* e: *Sense.* f: *Antisense. Hybridization signal was obtained with the sense probe* (arrowheads *in* e), *but little or no signal was obtained with the antisense probe. Methyl green counterstain; original magnification, ×400.*

consistent with the observation that TGF-β_3 is more potent than TGF-β_1 in stimulating collagen production by fetal rat bone cells[35] and may reflect differing receptor affinities for TGF-β_{1-3}.

Using digoxigenin-labeled riboprobes, we localized TGF-β isoform gene expression in normal murine lung and during bleomycin-induced lung fibrosis. In normal lung, TGF-β_1 and TGF-β_3 mRNA transcripts were identified in a wide variety of cells including bronchiolar epithelium, alveolar macrophages, alveolar type II cells, and mesenchymal and mesothelial cells. The widespread distribution of TGF-β_1 and TGF-β_3 mRNA transcripts in adult murine lung adds significant new information to previous data using radiolabeled probes[30] that showed gene expression for TGF-β_{1-3} to be limited to smooth muscle cells and fibroblasts in the bronchioles. These findings suggest that digoxigenin-labeled riboprobes are a more sensitive tool for cytokine detection than radiolabeled ones. They also suggest that TGF-β_1 and TGF-β_3 play important roles in normal lung homeostasis consistent with the recognized regulatory effects of TGF-β_1 on epithelial cell proliferation and differentiation,[36] immunomodulation,[37] and matrix protein turnover.[22]

A striking finding 10 days after bleomycin was the shift from predominant signal in airway epithelium of normal animals to predominant signal in alveolar walls. This may reflect both epithelial cell damage and inflammatory cell influx. It was not seen in control animals, indicating that it was specific to bleomycin injury. After bleomycin, TGF-β_1 gene expression was mainly localized to inflammatory cells including macrophages. Macrophages are recognized as an important source of TGF-β_1 in pulmonary fibrosis.[4,8] However, we have shown that signal in mesenchymal, alveolar type II, and mesothelial cells was also enhanced after bleomycin. Furthermore, the generalized increase in alveolar wall signal suggests that alveolar epithelial, microvascular endothelial, and subendothelial cells were also expressing TGF-β_1.

Mesenchymal cells expressing TGF-β_1 may be fibroblasts or smooth muscle cells. Lung fibroblasts produce TGF-β *in vitro*[38] and auto-induction may occur.[39] Fibroblast and endothelial cell TGF-β_1 gene expression increases after *in vitro* bleomycin exposure,[40,41] and TGF-β_1 augments endothelial matrix protein synthesis.[42] In the light of this evidence, our data suggest that increased TGF-β_1 gene expression by mesenchymal cells and pulmonary and capillary endothelial cells after bleomycin administration contributes to interstitial matrix accumulation.

Cultured mesothelial cells express TGF-β_1 mRNA,[43,44] but to our knowledge, ours is the first study to show this *in vivo*. Mesothelial cells have not hitherto been shown to express TGF-β_3 mRNA. We have previously demonstrated procollagen gene expression in the subpleural region of fibrotic mouse lung.[25,29] Mesothelial cells may thus participate in the pathogenesis of pulmonary fibrosis by producing TGF-β_1, which stimulates subpleural fibroblast collagen synthesis. TGF-β_1 also stimulates mesothelial

cell proliferation *in vitro;*[45,46] TGF-β_1 produced by these cells may therefore also act in autocrine fashion to stimulate their replication after injury. Our findings strengthen previous evidence suggesting that mesothelial cells participate in repair after pleural injury.[47–49]

Cells lining alveolar walls expressing TGF-β_1 may be alveolar type II cells or adherent macrophages. Bleomycin induces proliferation and metaplasia of type II cells.[50] TGF-β_{1-3} inhibit alveolar type II cell replication, and TGF-β secretion by these cells after bleomycin, as measured by bioassay, is inversely related to their proliferation.[20] TGF-β_1 production by metaplastic type II cells may therefore act in an autocrine manner to regulate their proliferation and differentiation after injury.

The time course for TGF-β_1 gene expression observed in this study using *in situ* hybridization is similar to that observed in previous studies that quantified TGF-β_1 mRNA by Northern analysis.[3,51,52] TGF-β_1 gene expression increased to a maximum around 10 days and then declined, with the pattern of expression returning to that seen in control animals by 35 days.

In contrast to TGF-β_1, TGF-β_3 gene expression was not enhanced after bleomycin nor associated with macrophage influx. Little signal was detected in bronchiolar epithelium or inflammatory cells after bleomycin. Gene expression of TGF-β_1 and TGF-β_3 is therefore differentially regulated, suggesting that TGF-β_1 but not TGF-β_3 is implicated in the pathogenesis of bleomycin-induced pulmonary fibrosis. Our findings are consistent with *in vitro* studies that have shown that TGF-β_1 but not TGF-β_2 or TGF-β_3 secretion by alveolar macrophages and alveolar epithelial type II cells increases during the evolution of this disease.[18,20] Furthermore, increased mRNA for TGF-β_1 but not TGF-β_2 has been demonstrated in bleomycin-treated mice.[19] In contrast, one study of bleomycin-induced pulmonary fibrosis has proposed that TGF-β_{1-3} are all implicated in the pathogenesis of pulmonary fibrosis.[21]

The results obtained with the TGF-β_2 probes were unexpected. There are several potential explanations. First, the sense probe could be inserted in the opposite direction to that predicted within the vector. This possibility was excluded by restriction mapping with *Cla*I and dideoxynucleotide sequencing. Second, the sense probe could be hybridizing with an unrelated species. This is relatively unlikely given the sequencing results. Third, the sense probe could be more heavily labeled with digoxigenin than the antisense probe. This was excluded using a chemiluminescence assay to assess labeling. Finally, a naturally occurring antisense molecule may be present in lung tissue. A precedent is the demonstration of an endogenous TGF-β_3 antisense molecule in chick embryo heart during cardiac valve formation.[53] Its temporally controlled appearance suggests it may regulate TGF-β_3 production during development. Pelton and colleagues[30] were able to demonstrate TGF-β_2 mRNA using the same probes as ours, suggesting that the appearance of a natural antisense molecule in mice may be strain or age specific. Additional studies using Northern analysis of lung with the TGF-β_2 riboprobes are required to establish whether such an antisense molecule exists. If confirmed, the implications of an antisense RNA molecule regulating TGF-β_2 gene expression will be important not only for our understanding of regulation of its gene expression and its role in the pathogenesis of fibrosis but also for the design of future therapies directed at modifying TGF-β function in a variety of fibrotic disorders.

In summary, we have shown that TGF-β_1, TGF-β_2, and TGF-β_3 all stimulate fibroblast procollagen synthesis *in vitro*, TGF-β_3 being the most potent in this assay. TGF-β_3 also reduced intracellular procollagen degradation. TGF-β_1 and TGF-β_3 gene expression was localized to a wide diversity of cell types in normal murine lung. After bleomycin, TGF-β_1 gene expression was maximally enhanced at 10 days and predominantly localized to macrophages. TGF-β_3 gene expression was not enhanced after bleomycin. TGF-β_2 mRNA was not detectable at any stage using the antisense probe, but signal was obtained using the sense probe, suggesting the presence of a naturally occurring antisense molecule. Differential gene regulation of the isoforms during the course of bleomycin-induced pulmonary fibrosis is consistent with data emerging from other models of lung injury. Our results suggest that, whereas TGF-β_1 is implicated in the pathogenesis of bleomycin-induced pulmonary fibrosis, TGF-β_3 may not be. The role of TGF-β_2 remains unclear.

Our findings have important implications for the development of anti-TGF-β strategies in the treatment of fibrotic lung disorders. A number of such strategies have been proposed,[1,54] including the use of antisense molecules, soluble receptor antagonists, and antibodies. If our results are confirmed in patients, TGF-β_1 will become the key target for such therapies.

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