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Increased type I procollagen mRNA transcripts in the lungs of mice during the development of bleomycin-induced fibrosis

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Increased type I procollagen mRNA transcripts in the lungs of mice during the development of bleomycin-induced fibrosis. S. Shahzeidi, P.K. Jeffery, G.J. Laurent, R.J. McAnulty. ©ERS Journals Ltd 1994.

ABSTRACT: In this study, *in situ* hybridization has been used to investigate the localization of type I procollagen messenger ribonucleic acid (mRNA) in normal lung, and in the lungs of mice during the development of bleomycin-induced pulmonary fibrosis.

Lung fibrosis was induced by a single intratracheal instillation of bleomycin sulphate (6 mg·kg^{-1} body weight), and tissues examined at times up to 35 days thereafter. Tissue transcripts of α_2(I) procollagen mRNA were visualized after hybridization with ^{35}S-labelled riboprobes.

Hybridization signals were associated with alveolar interstitial cells throughout the normal lung, with additional areas of dense hybridization signals observed subpleurally. Three days following administration of bleomycin, there was no apparent change in the pattern of hybridization. By 10 days, foci of intense hybridization signals indicative of gene activation were observed associated with individual cells in the alveolar interstitium. At 21 days, the increase in hybridization signals appeared to be associated with greater numbers of cells rather than highly activated cells.

These results demonstrate that procollagen genes are normally expressed in the mouse lung, and that during bleomycin-induced pulmonary fibrosis hybridization signals increase, suggesting that both enhanced gene expression by individual cells and increased numbers of cells expressing the type I procollagen gene are involved in the pathogenetic mechanism.

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Types I and III collagens are the most abundant proteins in the adult lung. They are present in the interstitium of all parenchymal structures, where they have a structural role, but in addition they are involved in cell-cell interactions [1]. An ordered distribution of these proteins is critical to the balance which must be achieved between structural integrity and the capacity for gas exchange between alveolar and vascular compartments. Gas exchange is impaired in interstitial lung disease where there is excessive collagen deposition.

To examine the pathogenesis of pulmonary fibrosis, several different animal models have been developed, the most common of which involves intratracheal instillation of bleomycin (for review see [2]). Throughout life, collagens are being continually synthesized and degraded in the lung [3, 4]. A change in the balance of these processes can result in the increased deposition of collagens. In bleomycin-induced pulmonary fibrosis, collagen synthesis is increased and there is evidence that decreased degradation also plays a role [5–7]. It has also been shown that steady-state levels of messenger ribonucleic acid (mRNA) for type I and III procollagens are increased in bleomycin-induced pulmonary fibrosis [8–11]. However, there is little information on whether a selected population of individual cells is activated to produce more collagen, or if all potential collagen synthesizing cells are activated. One study using *in situ* hybridization has suggested that type III procollagen mRNA is increased in individual cells of affected areas, with other areas of the lung appearing normal [11], but there is no information on the localization of type I procollagen mRNA.

In this study, we have employed the technique of *in situ* hybridization to localise mRNA transcripts of type I procollagen in the lungs of normal mice, and mice examined during the development of bleomycin-induced pulmonary fibrosis. The results suggest that mRNA for type I procollagen is present in alveolar interstitial cells throughout the normal lung; that following administration of bleomycin there are focal areas where individual cells show a particularly high state of type I procollagen gene activation; and that these are localised in the alveolar interstitium.

Methods

Animals

Mice (strain B_6 D_2 F_1) aged 8–9 weeks, and weighing 24–26 g, were anaesthetized using Alphaxolon 0.9% w/v and Alphadolon 0.3% w/v (Glaxo, UK), given intraperitoneally at a dose of 75 ml·kg^{-1} body weight. Bleomycin (Lundbeck, UK) was then administered intratracheally in 0.05 ml of 0.14 M NaCl, at a dose of 6 mg·kg^{-1} body weight. Control groups received 0.05 ml of 0.14 M NaCl alone. Briefly, the trachea was intubated and the appropriate solution introduced using a microsyringe with a 25 gauge blunt-ended needle. A small volume of air (0.2 ml) was then quickly injected to flush the airways, followed by another 0.05 ml of 0.14 M NaCl and a further 0.2 ml of air. Groups of nine mice, six for biochemical analysis and three for studies of *in situ* hybridization, were killed three, 10, 21, and 35 days later, by an overdose of pentobarbitone, as described previously [12]. For studies of *in situ* hybridization, lungs were fixed by intratracheal instillation of freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) at a pressure of 25 cmH_2O. The trachea was ligated just caudal to the larynx and the contents of the thorax removed en bloc. After 4 h immersion in fixative, tissues were transferred to 15% sucrose in PBS, prior to dehydration and embedding in paraffin wax.

For measurement of collagen content, animals were killed and lungs perfused with 2 ml of 0.9% PBS at 4°C. Total lung collagen content was assessed by measuring hydroxyproline, determined spectrophotometrically after oxidation with chloramine-T and extraction of the toluene miscible product [13]. Lung collagen content was calculated assuming that lung collagen contains 12.2% w/w hydroxyproline, and expressed as mg collagen·lung^{-1}.

Tissue preparation

The *in situ* hybridization method was based on techniques described previously [11]. These methods and the modifications adopted here are described briefly below. Unless otherwise stated, all steps were performed at room temperature.

Five micron thick sections 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 alcohol washes of decreasing concentration, followed by immersion in 0.14 M NaCl and PBS prior to refixation with 4% paraformaldehyde. To optimise entry of the probe into cells, sections were treated with proteinase-K (20 µg·ml^{-1} in 50 mM Tris-HCl, 5 mM ethylenediamine tetraacetic acid (EDTA) (pH 7.5) buffer for 7.5 min before refixing. To decrease surface charge, slides were immersed in 0.1 M triethanolamine, followed by dehydration through a series of increasing concentrations of ethanol.

Preparation of the probe

For hybridization an 860bp complimentary deoxyribonucleic acid (cDNA) fragment was employed containing the promoter, proximal exon and part of the first intron of the mouse α_2(I) procollagen gene [14]. This construct was initially cloned in pBR322, and after large scale preparation using polyethylene glycol precipitation [15], the plasmid was digested with $EcoR_1$ and ligated into pGEM-3Z. To generate antisense and sense riboprobes, *in vitro* transcription was performed using T7 or SP6 ribonucleic acid (RNA) polymerases, respectively, in the presence of ^{35}S-uridine triphosphate (UTP) (Amersham International, UK). Solutions were then treated with ribonuclease (RNase)-free deoxyribonuclease (DNase) (Boehringer, Germany), yeast transfer ribonucleic acid (tRNA) added as a carrier and dithiothreitol (DTT) added to minimize oxidation of the probe. Probes were then alkaline hydrolysed for 45 min in buffer containing 80 mM sodium carbonate and 120 mM sodium bicarbonate, and then neutralised in a 200 mM acetate buffer. Labelled probes were separated from unincorporated nucleotides by loading on to a column of sterile sephadex G-50, preblocked with yeast RNA, and eluted with 10 mM Tris-HCl (pH 7.5) containing 5 mM EDTA and 0.1% sodium lauryl sulphate. Fractions containing radioactivity were combined and the probe precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol at -70°C for at least 30 min. After centrifugation, the pellet was resuspended in 10 mM DTT to achieve a concentration of 5×10^5 cpm·µl^{-1} of the probe.

In situ *hybridization*

Hybridization buffer, composed of 50% formamide, 300 mM NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM sodium monophosphate (pH 8.0), 10% dextran sulphate, 1× Denhardt's solution and 500 µg·ml^{-1} yeast RNA was mixed with the radiolabelled probe at a ratio of 9:1. The resulting hybridization solution (25 µl) was applied to each tissue section and covered with siliconized coverslips.

Sections were incubated for 16 h at 52°C in a chamber humidified with a solution of 50% formamide in 2× standard sodium citrate (SSC). The optimal incubation temperature (52°C) was determined from the melting point of the probe (T_m), based on the salt concentration in the hybridization solution and the base composition of the probe.

Posthybridization washing

Following hybridization, slides were washed in 2× SSC, 10 mM DTT at 50°C for 30 min, 50% formamide, 2× SSC, 10 mM DTT at 65°C for 20 min, and twice in 2× SSC at 37°C each for 10 min. Sections were then treated with a solution of 4× SSC containing 20 µg·ml^{-1} RNase-A followed by washes of 2× SSC at 37°C for 10 min and 0.1× SSC at 37°C for 15 min. Finally, sections

were dehydrated in a series of washes with alcohol containing 0.3 M ammonium acetate and air dried.

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Sections were dipped into a photographic emulsion (K_5, Ilford, UK) previously warmed to 42°C and diluted (1:1) with distilled water. Slides were allowed to dry in the dark for 2 h and then placed in a light-tight box with silica gel for 3 weeks at 4°C. The slides were then immersed in developer for 3.5 min (D-19, Kodak, UK), and into fixer for 2 min (Rapid fixer, Ilford, UK), followed by several quick rinses with distilled water. Sections were then counterstained with haematoxylin, dehydrated through a series of ethanol washes and mounted using cytoseal 60 media (BDH, UK).

Results

Lungs of control mice contained 1.2±0.1 mg collagen (mean±SEM). Changes in lung collagen content after a single intra-tracheal dose of bleomycin are shown in figure 1. Collagen contents of bleomycin treated animals were not diffe-rent from controls at 3 days, but by 10 days had increased by 40% ($p<0.05$). After this time, there was a further increase, and by 35 days the content was almost double that of controls ($p<0.01$).

Adjacent sections of normal lung tissue, hybridised with an antisense probe for α_2(I) procollagen and stained with haematoxylin and eosin are shown in figure 2a and b, respectively. Hybridization signals for type I procollagen mRNA were associated with cells within the alveolar interstitium and were visible throughout the lung. Clusters of hybridization signals were clearly visible over individual cells. Areas of higher grain density were observed in association with cells beneath the pleura (fig. 2a), and around major airways (not shown). Hybridization signals did not appear to be associated with epithelial cells. A similar pattern of hybridization was observed in lungs from saline control animals and throughout the 35 day time-course. Hybridization of normal lung sections with a sense probe showed very low levels of non-specific signals, similar to those shown in figure 3d for bleomycin treated animals.

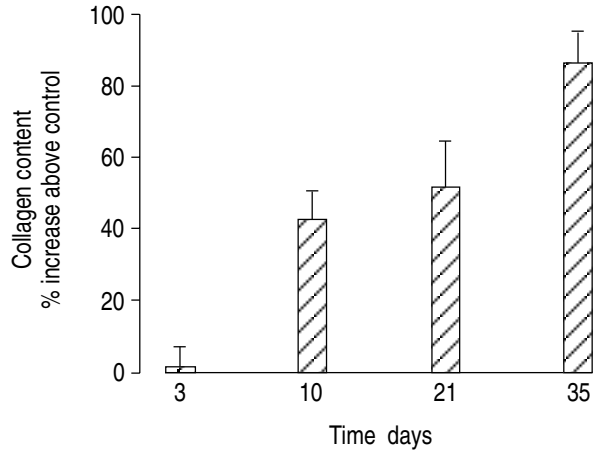

Fig. 1. – Changes in lung collagen content expressed as a percentage increase above control lung collagen content at each time following the intratracheal instillation of bleomycin. Values represent the mean±SEM (n=6).

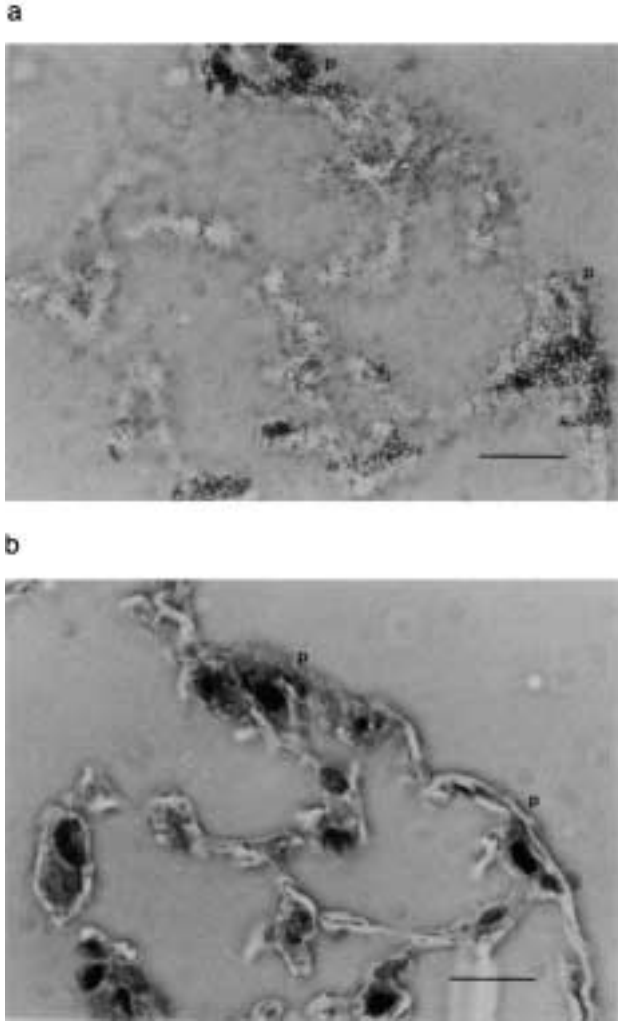

Fig. 2. – Serial sections of a normal mouse lung showing localization of α_2(I) procollagen messenger ribonucleic acid (mRNA): a) brightfield microscopy; and b) stained with haematoxylin and eosin. The antisense probe was used. Hybridization signals for type I procollagen mRNA, represented by the black dots, can be seen throughout the interstitial structures, with clusters of hybridization signals overlaying individual cells. Areas of higher grain density were observed associated with cells beneath the pleura (marked p). (Original magnification ×400; scale bar=50 µm).

Three days after instillation of bleomycin there were no obvious differences in the pattern of hybridization signals compared with controls. Ten days after administration of bleomycin, small areas of peribronchial inflammation spreading peripherally were observed, with a mild reactive cuboidal cell hyperplasia. The inflammatory cells consisted predominantly of mononuclear cells with fewer neutrophils. The presence of mRNA transcripts indicative of type I procollagen gene expression in the lung of an animal 10 days after intratracheal instillation of bleomycin are shown in figure 3. Bright and dark field views (fig. 3a and b) demonstrate areas of marked

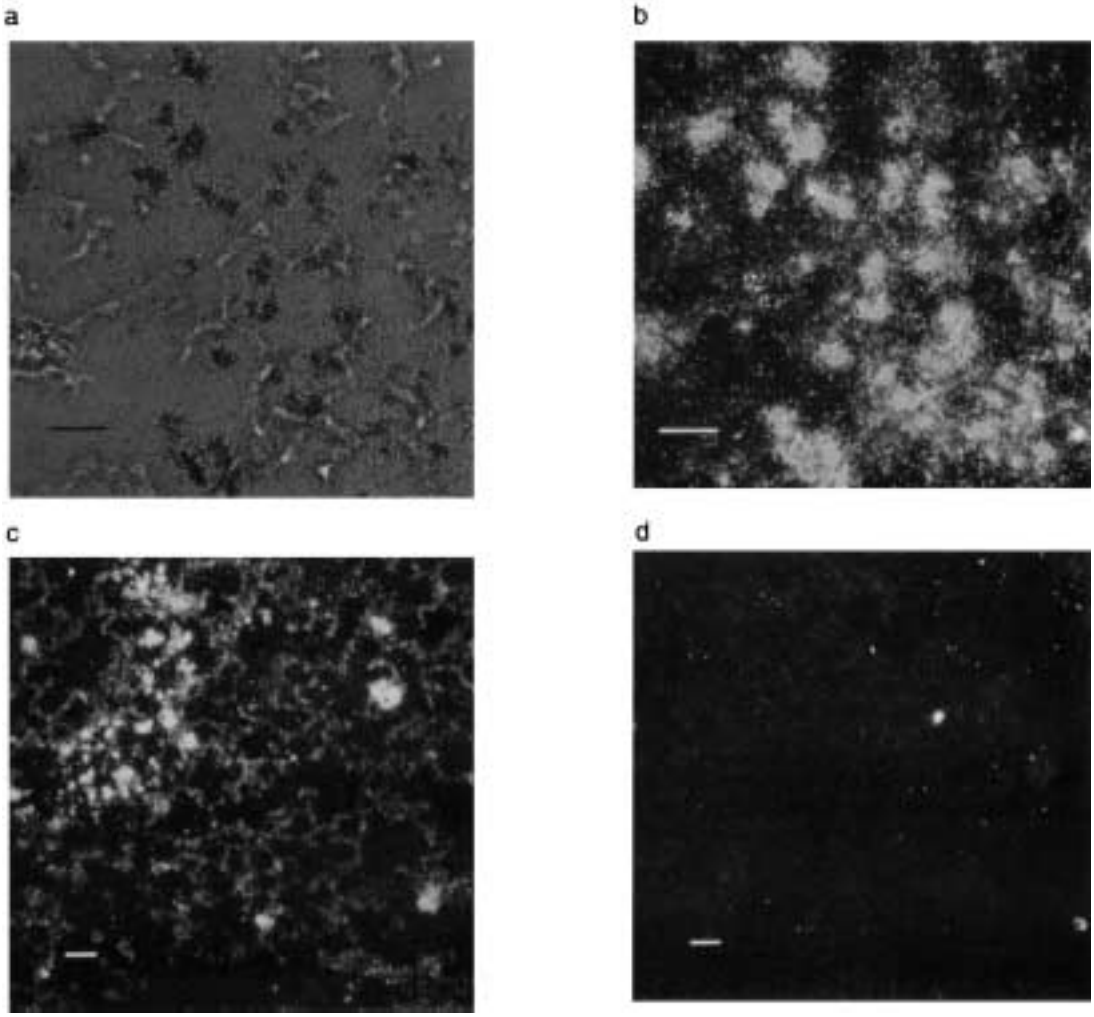

Fig. 3. – Localization of α_2 (I) procollagen messenger ribonucleic acid (mRNA) in lung from an animal 10 days after bleomycin treatment. Sections hybridized with the antisense probe are shown (a–c). An area of highly-activated cells with dense hybridization signals clustered above them is shown viewed by: a) brightfield microscopy; and b) darkfield microscopy. With darkfield microscopy the hybridization signals appear white, further highlighting the intense type I procollagen gene activity. c) A darkfield view of this area at lower magnification reveals the localized nature of the type I procollagen gene activation. Hybridization signals were detected throughout the surrounding alveolar interstitium but were more sparsely distributed. d) Application of sense probe demonstrated very limited nonspecific binding. (Brightfield (a) and darkfield microscopy (b–d)). (Original magnification ×200 (a and b), ×100 (c and d); scale bar=50 μm).

procollagen gene expression associated with individual cells in the lung interstitium. Patchy areas of gene activation were obvious throughout the lung, with other areas showing only slight or no visible increase in gene activity compared with controls (fig. 3c). Similar patterns of hybridization were observed in all sections examined at this time-point. Sections of lung hybridized with a sense probe showed low levels of nonspecific signals (fig. 3d).

By 21 days, the peribronchial lesions had increased in size, with thickened interstitium and loss of normal alveolar architecture, indicative of the development of fibrosis. In these areas, a higher overall density of hybridization signals was apparent (fig. 4) compared with those observed in the normal lung (fig. 2a). This was generally associated with the increased cellularity and loss of normal alveolar structure rather than increased type I procollagen mRNA transcripts in individual cells. Occasional intensely labelled cells were still present, as shown in the top right of figures 4b and c, but these were not as abundant as at the 10 day time-point and tended to be isolated rather than in focal groups. Overall, there appeared to be less mRNA transcripts for type I procollagen at 21 days than at 10 days following administration of bleomycin.

Thirty five days after a single dose of intratracheal bleomycin, there was histochemical evidence of fibrosis, with interstitial thickening and loss of normal alveolar architecture. Type I procollagen gene expression was limited to cells at the periphery of fibrotic foci, whilst, in apparently unaffected areas, the pattern of hybridization appeared similar to that in saline control lungs. No hybridization signals could be detected within the consolidated fibrotic foci.

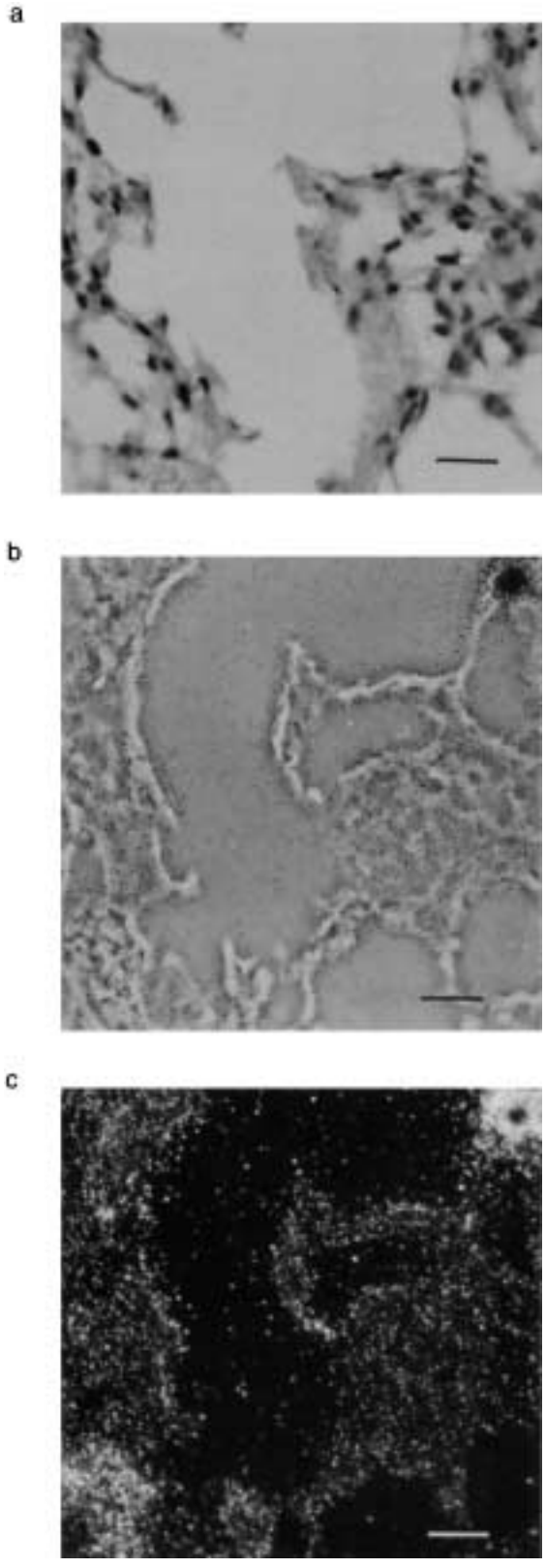

Fig. 4. – Serial sections of mouse lung 21 days after bleomycin treatment showing: a) peribronchial lesions with thickened interstitium; and localization of type I procollagen messenger ribonucleic acid (mRNA): b) brightfield; and c), darkfield. Hybridization signals were evenly distributed over the dense cellular lesions with few highly activated cells (top right). (Original magnification ×200; scale bar=50 μm).

Discussion

In the present study, we have demonstrated that the α_2(I) procollagen gene is expressed in normal lung tissue. Messenger RNA was detected at low levels in cells throughout the interstitium, with isolated foci of more active expression in subpleural regions and around major airways. This contrasts with previous studies in normal human lung, which have failed to detect type I procollagen mRNA [16]. The current study is, therefore, consistent with biochemical evidence suggesting that collagen is continually synthesized in the lung [17, 18]. However, it is known that lung collagen synthesis rates decrease with age [3, 4] and it is, therefore, likely that in adult humans and in older animals the levels of mRNA for type I procollagen would fall below the threshold level detectable by *in situ* hybridization. Indeed, in recent studies examining type III collagen, which is present in lower amounts than type I collagen, we were only able to detect type III procollagen mRNA in subpleural areas of normal lung [11], the area where greatest activity was observed using the α_2(I) procollagen probe in this study.

After intratracheal instillation of bleomycin, the collagen content of the lung, assessed by measurement of hydroxyproline, increased by about 40% within 10 days. Thereafter, collagen continued to increase, and by 35 days was double that of controls. Histochemically, there was evidence of peribronchial inflammation spreading peripherally at 10 and 21 days, but there was no evidence of interstitial fibrosis until 35 days. These results are similar to those described previously in this model [12].

Type I procollagen gene expression was also increased by 10 days. In some regions of the lung there were many very highly activated cells, whereas other areas of the sections showed only slight or no visible increase in gene activity. Twenty one days after treatment with bleomycin, hybridization signals were apparent throughout the active lesions, with far fewer highly activated individual cells. By 35 days, consolidated fibrotic areas had appeared in which very few hybridization signals were apparent, but around the periphery of these and in unaffected areas of the lung hybridization was still visible. The time scale for expression of type I procollagen mRNA shown here by *in situ* hybridization is similar to that observed previously by measuring total type I procollagen mRNA [8–10], but in the present study we were able to demonstrate further that gene activation is highly localised, to specific areas of active disease (fig. 3 and 4). This would suggest that the mechanisms involved in controlling procollagen gene activation are also highly localized, and provides circumstantial evidence for the local production of autocrine or paracrine regulatory mediators. This concept is supported by several studies which suggest that transforming growth factor β_1, a potent stimulant of collagen production, co-localizes with the deposition of collagens in areas of active fibrosis both in human pulmonary fibrosis [16, 19, 20], and bleomycin-induced pulmonary fibrosis [21].

In the present study, the peak of expression for type I procollagen appeared to occur around 10 days. In contrast, a previous study of type III procollagen gene

expression by *in situ* hybridization demonstrated peak activation later, at about 21 days after administration of bleomycin [11]. In this study, the pattern of hybridization for type III procollagen at 21 days was similar to that shown here for type I procollagen at 10 days. Similar differences in the time-course for procollagen gene activation have been reported from measurements of total mRNA [8, 10]. These data suggest that there is differential regulation of collagen types during the development of pulmonary fibrosis.

The activated cells are difficult to identify due to the overlay of autoradiographic grains produced by the hybridization protocol. However, it is generally accepted that the fibroblast is the major collagen producing cell in the lung, and the interstitial location of hybridization signals observed would be consistent with this view.

Cells which were highly activated in terms of type I procollagen gene expression were located almost exclusively in areas of early active disease. In the fibrotic foci, very little type I procollagen mRNA could be detected, with only a few active cells at the periphery of the lesions. Similar observations have been reported previously by us for type III procollagen gene expression in bleomycin-induced pulmonary fibrosis [11], and in studies of α_1(I) procollagen gene expression in lung sections from patients with idiopathic pulmonary fibrosis [16].

In summary, we have demonstrated that type I procollagen mRNA is present throughout the normal lung. After administration of bleomycin intratracheally, increased α_2(I) procollagen gene expression was initially associated with localized areas of highly activated individual cells. Whilst, at later times, gene activation appeared to be associated mainly with increased cellularity. In contrast, consolidated fibrotic foci which appeared at the latest times had very little type I procollagen gene activity.

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