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Diverse cellular TGF-β_1 and TGF-β_3 gene expression in normal human and murine lung

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ABSTRACT: A role for transforming growth factor-beta$_1$ (TGF-β_1) has been proposed in lung development and in the pathogenesis of pulmonary disease. However, previous studies have not delineated the cells expressing TGF-β_1 in normal adult lung, nor compared its gene expression with that of other TGF-β isoforms.

We used digoxigenin-labelled riboprobes to localize TGF-β_1 and TGF-β_3 gene expression in normal adult human and mouse lung.

This procedure was technically simple, providing excellent resolution. TGF-β_1 and TGF-β_3 messenger ribonucleic acid (mRNA) transcripts were detected in a wide variety of cells. In human lung, mRNA for both isoforms was localized to bronchiolar epithelium and alveolar macrophages. TGF-β_1, but not TGF-β_3 mRNA was detected in mesenchymal and endothelial cells. In murine tissue, TGF-β_1, mRNA was localized to bronchiolar epithelium, Clara cells, mesenchymal cells, pulmonary endothelium and alveolar cells, including macrophages. TGF-β_3 mRNA was similarly distributed but not detected in endothelium.

In summary, using a nonisotopic technique in lung tissue, we have detailed the cells expressing the transforming growth factor-β_1 and β_3 genes in human and murine lung. There was widespread expression of these cytokines in normal lung consistent with autocrine or paracrine roles in regulating cellular turnover, immune defence and matrix protein metabolism.

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The role of cytokines in lung development and in the pathogenesis of pulmonary disease is central to our understanding of normal and abnormal lung physiology. One cytokine of interest in the lung is transforming growth factor-beta$_1$ (TGF-β_1), a 25 kDa homodimeric polypeptide with multiple functions, including regulation of cellular proliferation and differentiation, immune function, and extracellular matrix metabolism. It is thought that TGF-β_1 plays a role in the pathogenesis of asthma [1], malignancy [2, 3], and various interstitial lung diseases, including those complicated by fibrosis [4, 5].

However, the existence of at least three mammalian TGF-β isoforms is now recognized. Gene expression and protein synthesis for all three have been documented in embryonic and adult murine lung [6–8], but relatively little is known about the precise cells expressing these genes. Gene expression of TGF-β isoforms has not been studied in the normal human lung. *In vitro* studies suggest that the three isoforms have similar biological activities [9], but their roles *in vivo* remain unclear. In skin, it has been suggested that they may have very different functions during wound healing, TGF-β_1 promoting scarring, while TGF-β_3 acts as an antiscarring agent [10]. Detailed studies of the sites of gene expression for the different isoforms may provide additional clues on this issue.

Up to the present time, most studies of gene expression in the respiratory tract have employed semiquantitative Northern blot analysis of total lung ribonucleic acid (RNA), or have examined localization using *in situ* hybridization with radiolabelled probes. Our laboratory has experience with the latter technique [11, 12], but these studies are time-consuming and resolution of tissue structures and cellular morphology tends to be poor. The lung contains many different cell types [13], and the development of methods which provide good resolution is, therefore, critical for the identification of cells expressing the gene product in question.

In this study, digoxigenin-labelled riboprobes were synthesised, and used to localize TGF-β_1 and TGF-β_3 mRNA transcripts in normal human and murine lung tissue.

We demonstrate that digoxigenin-labelled riboprobes provide excellent resolution and preservation of cellular morphology. We show that TGF-β_1 and TGF-β_3 mRNA transcripts are distributed in a wide variety of lung cells, suggesting that both isoforms play important roles in normal lung homoeostasis.

Materials and methods

Tissue selection and preparation

Lung tissue was obtained from six healthy, 8 week old mice (strain $B_6D_2F_1$) and from six patients undergoing

resection for lung cancer. There were equal numbers of males and females, aged 44–59 yrs. They were diagnosed as having bronchial adenocarcinoma and were present or past smokers. Only macroscopically and histologically normal peripheral lung was selected for these studies. Viable tissue was fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS). After 4 h immersion in fixative, lung tissue was transferred to 15% sucrose in PBS prior to dehydration and embedding in paraffin wax.

Prehybridization treatments were performed using techniques described previously [11, 14]. Five micron sections were mounted onto 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 M sodium chloride and PBS before refixing in freshly prepared 4% paraformaldehyde. To maximize probe penetration, sections were treated with proteinase K (Life Technologies, Paisley, UK) at a concentration of 20 $\mu g \cdot mL^{-1}$ in 50 mM Tris-HCl (pH 7.5), 5 mM ethylenediamine tetra-acetic acid (EDTA) for 10 min before refixing with paraformaldehyde. In order to reduce electrostatic binding of the probe, slides were acetylated by immersion in freshly prepared 0.1 M triethanolamine containing 0.25% acetic anhydride. Sections were subsequently dehydrated through a series of increasing concentrations of ethanol.

Probe preparation

The templates for riboprobe synthesis were transcript-specific murine TGF-β_1 and TGF-β_3 complementary deoxyribonucleic acid (cDNA) constructs in pGEM® vectors Promega, UK. They were obtained by deleting the highly conserved regions of the two murine cDNAs, and the specificity of riboprobes synthesized from this template is well-documented [8, 15]. The TGF-β_1 construct includes nucleotides 421 to 1395 of the murine deoxyribonucleic acid (DNA), and contains 764 base pairs (bp) of the N-terminal glycopeptide (precursor) region and 210 bp of the mature region. The TGF-β_3 construct contains 609 bp of the N-terminal glycopeptide region (831 to 1440).

Digoxigenin-labelled riboprobes were synthesized by *in vitro* transcription of 1 µg of appropriately linearized template using SP6 (sense) or T7 (antisense) according to the manufacturer's instructions (Boehringer Mannheim, Lewes, UK). Chemiluminescence assay (Boehringer Mannheim) of TGF-β_1 antisense riboprobe confirmed successful labelling.

To determine the optimum probe length for *in situ* hybridization, limited alkaline hydrolysis of the TGF-β_1 antisense riboprobe was performed using a modification of a method published previously [16]. Incubation of the TGF-β_1 probe with alkali yielded fragments of approximately 80 bp length, as measured by agarose gel electrophoresis and comparison with nucleic acid molecular size standards. The results of using unhydrolysed probe were compared with those using hydrolysed probe.

In situ *hybridization*

This protocol was based on previously published methods using radiolabelled probes [12]. Hybridization buffer consisting of 50% deionized formamide, 300 mM NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM monosodium phosphate (pH 8.0), 10% dextran sulphate, 1× Denhardt's solution and 500 $\mu g \cdot mL^{-1}$ brewer's yeast transfer ribonucleic acid (tRNA) was mixed with digoxigenin-labelled probe at a ratio of 9:1, to give a final probe concentration of 20 $ng \cdot mL^{-1}$. Twenty five microlitres of hybridization solution was applied to each slide and the mixture covered with siliconized coverslips. Sections were incubated overnight at 50°C in a sealed chamber humidified with a solution of 50% formamide in 2 × standard sodium citrate (SSC). Following hybridization, the slides were washed in 4 × SSC for 30 min and then in 0.2 × SSC for 30 min at room temperature.

Detection of hybridized probe

Slides were washed in Tris buffered saline (TBS; consisting of 0.1 M Tris (pH 8.2), 0.15 M NaCl) for 5 min. This and subsequent incubations were performed at room temperature. They were then incubated for 30 min with an antibody blocking solution consisting of 5% (w/v) bovine serum albumin (BSA) and 5% (v/v) normal sheep serum diluted in TBS with 0.1% (v/v) Tween 20®. After two further 5 min washes in TBS the slides were incubated with antibody solution for 30 min. This consisted of a 1:100 dilution of anti-digoxigenin-alkaline phosphatase, Fab fragments (Boehringer Mannheim) in 1% BSA in TBS with Tween 20®. Sections were then washed twice in TBS, for 5 min each.

For detection of bound antibody, sections were incubated with an alkaline phosphatase substrate, New Fuschin Red (Dako, UK). This was prepared according to the manufacturers' instructions. Briefly, three drops each of chromogen (0.5 % New Fuschin Red in HCl) and activating agent (in aqueous solution) were mixed in a 5 mL tube and allowed to stand for 3 min. Buffered substrate (naphthol phosphate in Tris buffer) containing 1 mM levamisole to inhibit endogenous alkaline phosphatase activity [17] was added to a final volume of 2 mL and the solution mixed gently. Two hundred microlitres of this freshly prepared reagent was applied to each section and incubated for 20 min at room temperature.

The slides were then rinsed in distilled water and counterstained with haematoxylin or methyl green. They were air-dried and mounted in glycerol without dehydration. New Fuschin Red yields a permanent, non-alcohol-fast red colour at the site of bound antibody. It also stains extracellular matrix proteins a faint pink. Sections were examined and reported upon independently by three of the authors (RKC, PKJ and RJM).

Results

Validation of results

Figure 1 shows sections of: mouse lung hybridized with antisense (fig. 1a) or sense (fig. 1b) TGF-β_1 probe;

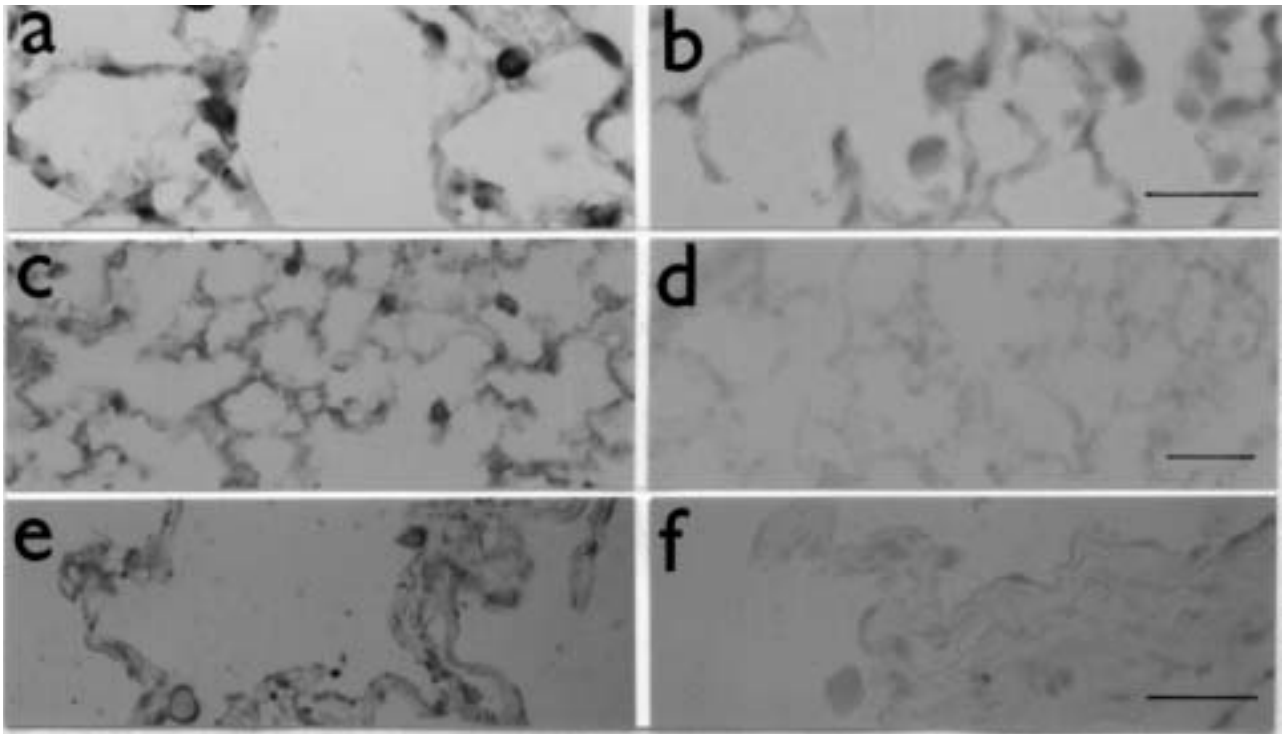

Fig. 1. – Sense and antisense controls for TGF-β_1 and TGF-β_3 riboprobes. a–b) Murine lung hybridized with TGF-β_1 probe: a) antisense; b) sense. c–d) Murine lung hybridized with TGF-β_3 probe: c) antisense; d) sense. e–f) Human lung hybridized with TGF-β_1 probe: e) antisense; f) sense. The hybridization signal appears as a red colour at the site of mRNA detection. TGF: transforming growth factor; mRNA: messenger ribonucleic acid. (Original magnification ×400 (a,b,e,f) and ×200 (c,d); internal scale bar=50 µm.

mouse lung hybridized with antisense (fig. 1c) or sense (fig. 1d) TGF-β_3 probe; and normal human lung tissue hybridized with antisense (fig. 1e) or sense (fig. 1f) TGF-β_1 probe. In human lung, the distribution of TGF-β_3 mRNA transcripts was similar to that of TGF-β_1 and this data is, therefore, not shown.

There was good discrimination between sense and antisense probes, with an absence of signal in sections to which the sense probe was applied. In all cases, hybridization signal was clearly cytoplasmic and outlined the nucleus. There was faint pink staining of extracellular matrix proteins by New Fuschin Red but this did not interfere with detection of hybridization signal. There was no increase in signal intensity using hydrolysed

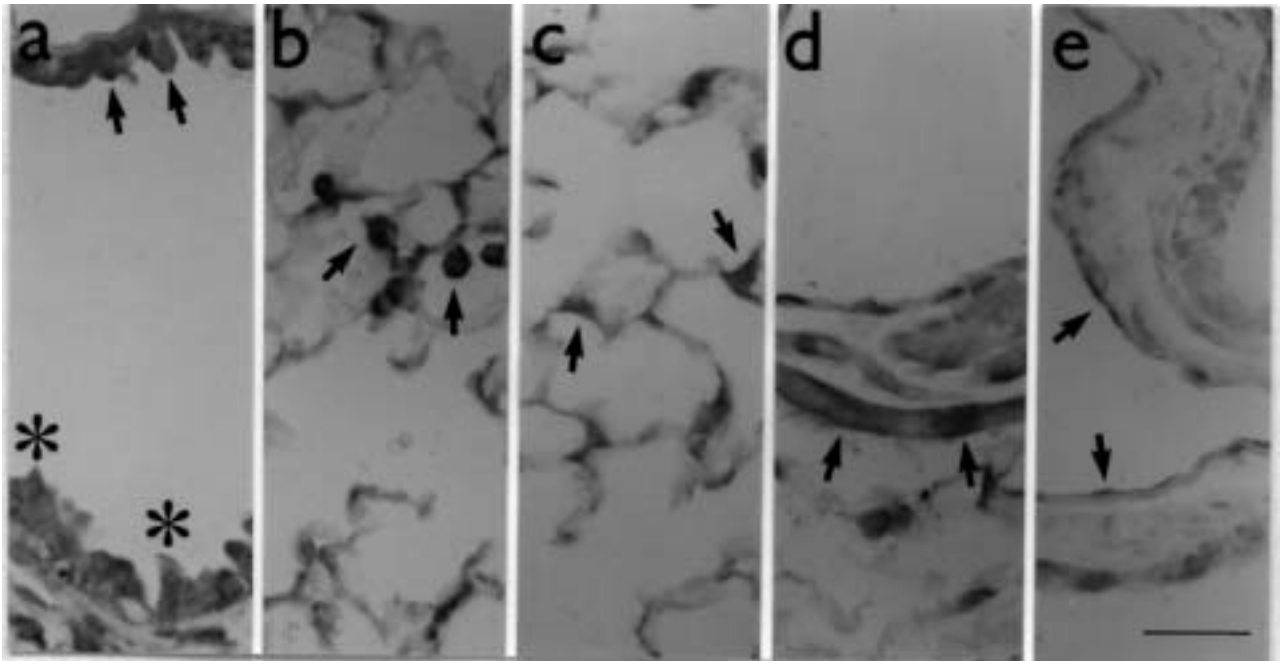

Fig. 2. – Localization of TGF-β_1 mRNA in murine lung. a) Bronchiolar epithelial cells (arrows) including Clara cells (asterisks); b) alveolar macrophages; c) cells lining alveolar walls; d) mesenchymal cells underlying a blood vessel; e) pulmonary endothelial cells. TGF-β_1: transforming growth factor-β_1; mRNA: messenger ribonucleic acid. (Original magnification ×400; internal scale bar=50 µm).

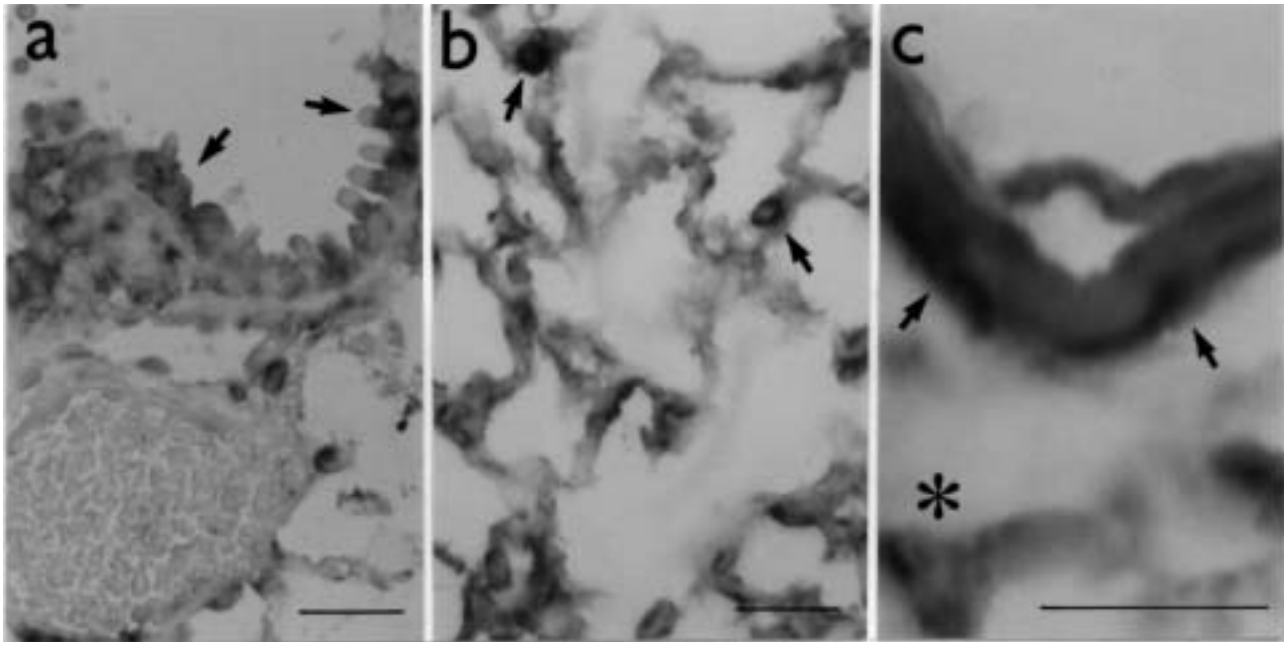

Fig. 3. – Localization of TGF-β_3 mRNA in murine lung. a) Bronchiolar epithelial cells; b) alveolar macrophages; c) mesenchymal cells (arrows) and cells lining alveolar walls (asterisk). TGF-β_3: transforming growth factor-β_3; mRNA: messenger ribonucleic acid. (Original magnification ×400 (a, b) and ×1,000 (c); internal scale bar=50 μm).

probe compared with that using unhydrolysed probe (data not shown). Unhydrolysed probe was, therefore, used in all subsequent studies.

Cellular localization of TGF-β mRNA transcripts in murine lung

Figure 2 shows the localization of hybridization signal for TGF-β_1 in murine lung. It was predominant in bronchiolar epithelial cells, including Clara cells (fig. 2a) and in luminal alveolar macrophages (fig. 2b). However, it was also present in cells lining alveolar walls (fig. 2c), mesenchymal cells underlying blood vessels (fig. 2d) and pulmonary endothelial cells (fig. 2e). The cells lining alveolar walls were identified as alveolar type II cells or macrophages adherent to the alveolar wall. The mesenchymal cells are likely to be fibroblasts but this was not proved.

Figure 3 shows the cell types expressing TGF-β_3 mRNA. The distribution of TGF-β_3 mRNA transcripts

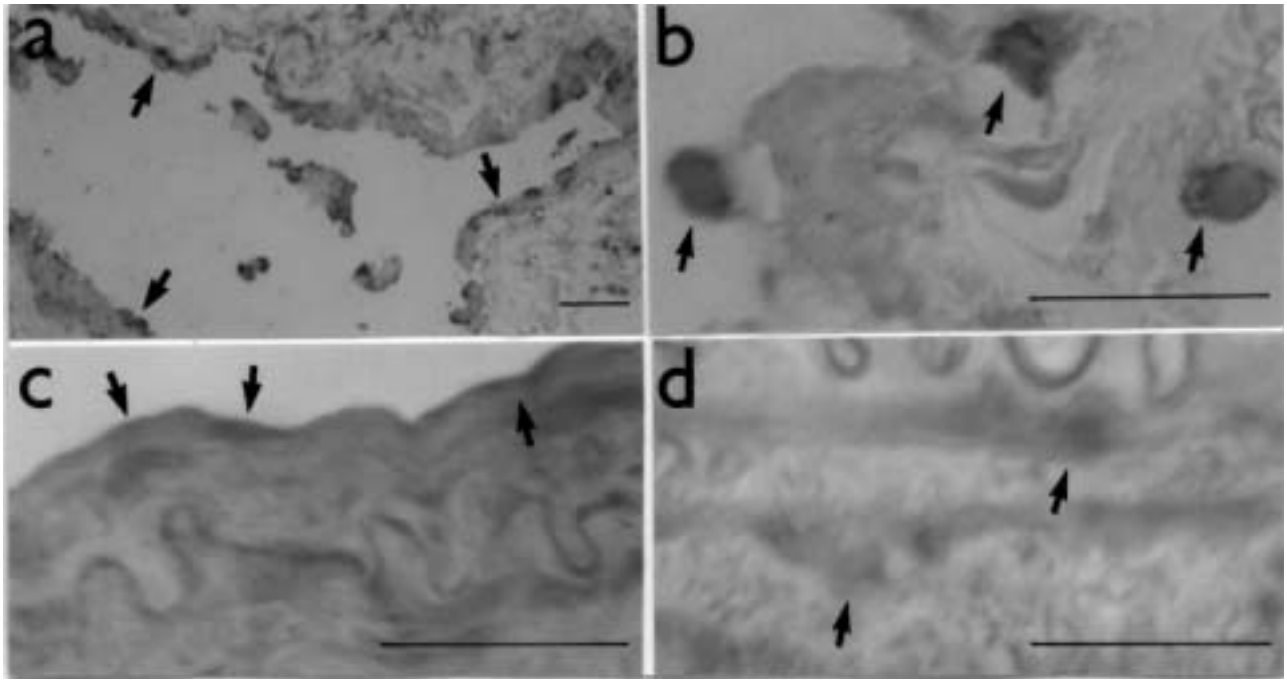

Fig. 4. – Localization of TGF-β_1 mRNA in human lung. a) Bronchiolar epithelial cells; b) alveolar macrophages; c) pulmonary endothelial cells; d) mesenchymal cells. TGF-β_1: transforming growth factor-β_1; mRNA: messenger ribonucleic acid. (Original magnification ×200 (a) and ×1,000 (b, c and d); internal scale bar=50 μm).

in murine lung was similar to that of TGF-β_1. Hybridization signal was again predominant in bronchiolar epithelial cells (fig. 3a) and alveolar macrophages (fig. 3b), but was also detected in mesenchymal cells underlying blood vessels and cells lining alveolar walls (fig. 3c). Messenger RNA transcripts were not detected in pulmonary endothelial cells.

Cellular localization of TGF-β mRNA transcripts in human lung

Hybridization signal was generally less intense in human lung than in murine lung but was again localized to a wide variety of cell types (fig. 4). TGF-β_1, mRNA transcripts were predominantly localized in bronchiolar epithelial cells (fig. 4a) and in alveolar macrophages (fig. 4b), but were also detected in pulmonary endothelial cells (fig. 4c) and mesenchymal cells (fig. 4d). As in murine lung, these are likely to be fibroblasts.

The distribution of TGF-β_3 mRNA transcripts was similar to that of TGF-β_1. Hybridization signal was present in bronchiolar epithelial cells and alveolar macrophages (data not shown). However, TGF-β_3 mRNA transcripts were not detected in mesenchymal or pulmonary endothelial cells in human lung.

Discussion

Use of digoxigenin-labelled riboprobes

Up to the present time, few studies have employed digoxigenin-labelled probes in the respiratory tract. We have found that synthesis of digoxigenin-labelled riboprobes is reliable and efficient. RNA yield is excellent, digoxigenin incorporation predictable and the technique is simple to perform. Hydrolysis of probes to 50–500 bp is sometimes recommended [18], shorter probes being thought to penetrate more easily into cross-linked tissues than longer ones. In our study, signal was not enhanced by probe hydrolysis. This suggests that if lung tissue is sufficiently permeabilized, hydrolysis of probes not exceeding 1,000 bp is unnecessary. The longer the probe, the greater the number of digoxigenin molecules incorporated. Antibody binding is correspondingly amplified and signal intensity enhanced. We would, therefore, recommend that the longest probes which can reproducibly penetrate the tissue are used to achieve maximum sensitivity. Digoxigenin-labelled oligonucleotides may consequently not be appropriate for detecting mRNA transcripts present in low copy number in lung tissue.

Nonisotopic methods offer technical advantages over radioactive protocols, including safety of handling and waste disposal, the facility for long-term storage of probe, and speed of execution. The procedure presented here can be completed within 48 h. A further advantage is enhanced tissue resolution. Comparison of the present results with previous *in situ* hybridization studies in the literature [8, 11, 12] suggests that gene expression can be more precisely localized in lung tissue using a non-radioactive technique. The clearly cytoplasmic nature of hybridization signal illustrates its specificity and the high degree of resolution obtained. Furthermore, we observed very little nonspecific signal. This is important because high levels of nonspecific binding seen with radiolabelled probes may confound identification of labelled cells. We chose to use the Dako reagent because preliminary experiments with alternative alkaline phosphatase substrates yielded colours which faded and became granular in appearance with time (unpublished observations). The advantages conferred by the use of digoxigenin-labelled riboprobes are illustrated by the findings discussed below.

Cellular localization of TGF-β_1 and TGF-β_3 mRNA transcripts in normal lung

We have shown that mRNA transcripts for TGF-β_1 and TGF-β_3 are present in adult murine lung and predominantly localized to bronchiolar epithelial cells, including Clara cells, and alveolar macrophages. They are also present in mesenchymal and alveolar lining cells. TGF-β_1 mRNA transcripts are also observed in pulmonary endothelial cells. Relatively few large blood vessels were present in the sections of mouse lung examined; this may account for TGF-β_3 mRNA transcripts not being detected in pulmonary endothelium. Messenger RNA transcripts for both TGF-β isoforms are present in normal human lung and predominantly localized to bronchiolar epithelium and alveolar macrophages. TGF-β_1 gene expression is also observed in pulmonary endothelial and mesenchymal cells.

In an earlier study, PELTON *et al*. [8] used ^{35}S-labelled probes to localize TGF-β isoform gene expression in murine lung. They demonstrated TGF-β_1 and TGF-β_3 gene expression in mesenchymal cells underlying bronchiolar epithelium, but did not detect signal in bronchiolar epithelial, alveolar or endothelial cells. A study in sheep lung also failed to demonstrate TGF-β mRNA transcripts in bronchiolar epithelium [15]. Both these studies used riboprobes of identical sequence to those used in the present study. In human lung TGF-β_1 gene expression has so far only been documented in alveolar macrophages [4]. As far as we are aware, this study is the first to document TGF-β_3 gene expression in human lung. Endothelial cells express TGF-β_1 mRNA and secrete TGF-β_1 *in vitro* [19], but TGF-β_1 gene expression by endothelial cells *in vivo* has not, to our knowledge, been reported previously. Taken together, these data suggest that digoxigenin-labelled riboprobes may be more sensitive than ^{35}S-labelled riboprobes. Certainly, a recent report showed that digoxigenin-labelled riboprobes are at least as sensitive as ^{35}S-labelled riboprobes when used to detect interleukins in T-cells [20].

Immunostaining shows the TGF-β isoforms to be preferentially localized in bronchiolar epithelial cells in murine lung [8]. These authors suggested that the presence of TGF-β protein but not mRNA transcripts in bronchiolar epithelium indicated a paracrine mode of action for this peptide. We propose instead that gene expression and synthesis of TGF-β_1 and TGF-β_3 are co-localized in bronchiolar epithelial cells, permitting autocrine as well as paracrine modes of action. The present findings in human lung are consistent with those of

MAGNAN *et al.* [21] who showed TGF-β protein preferentially localized to bronchial epithelium.

Human lung tissue was taken from current or previous smokers, raising the possibility that smoking might alter TGF-β gene expression. No studies have yet addressed this question directly. However, in a study of TGF-β_1 gene expression in human airways [22], no difference was found in TGF-β_1 gene expression between smokers and nonsmokers. Furthermore, we made similar observations in mice with no exposure to smoke. We therefore think it is unlikely that smoking induced TGF-β mRNA transcripts in cells not previously identified as expressing these genes.

Possible functions of TGF-β_1 and TGF-β_3 in normal lung

The wide variety of cells in the lung expressing the TGF-β_1 and TGF-β_3 genes suggests that these peptides have important roles in normal lung homeostasis. Multiple *in vitro* biological effects are ascribed to TGF-β_1, including regulation of cell proliferation, immune response and extracellular matrix metabolism. TGF-β_1 inhibits epithelial [23] and endothelial cell proliferation [24, 25] *in vitro*, and transformed bronchial epithelial cells lose their sensitivity to growth inhibition by TGF-β_1 [26]. In normal mature lung, TGF-β_1 and TGF-β_3 may, therefore, act in autocrine fashion in bronchiolar epithelial and pulmonary endothelial cells to maintain physiological homeostasis by regulating cell proliferation and subsequent differentiation. Clara cells can participate in epithelial repair by division and differentiation [27], and type II cells perform a similar function in the alveoli [28], thus, possibly explaining why these cell types express TGF-β.

TGF-β may also play a role in normal immune defence. Activated human macrophages express TGF-β_1 mRNA [29], and constitutive TGF-β_1 gene expression by alveolar macrophages in normal human lung has been documented [30]. This may reflect continuous macrophage activation, also exhibited by nonspecific pathogen-free mice. TGF-β_1 has various effects on macrophage function, including inhibition of hydrogen peroxide production and cytotoxicity [31]. TGF-β gene expression by macrophages in normal lung may indicate a vital role in preventing tissue injury following repeated exposure to inhaled irritants and pathogens. Certainly animals in which TGF-β_1 expression is compromised succumb early in life to a fatal multifocal inflammatory disease [32]. In bronchiolar epithelial cells, TGF-β may have a further immunomodulatory role consistent with the ability of TGF-β_1 to stimulate immunoglobulin A (IgA) secretion by β-lymphocytes [33]. Finally, TGF-β is also a potent stimulator of extracellular matrix protein synthesis [34]. Collagen, the major extracellular matrix protein in lung, is synthesized and degraded throughout life [35]. TGF-β produced by bronchiolar epithelial, pulmonary endothelial and mesenchymal cells may, therefore, play a further role in regulating extracellular matrix metabolism.

In summary, we have described a method using digoxigenin-labelled riboprobes to localize cytokine gene expression in lung tissue. We have shown that this technique is sensitive, quick and reproducible. It offers technical advantages over isotopic methods and provides improved tissue resolution, with a greater potential for specific signal localization. Using this technique, we have identified a wide variety of lung cells not previously recognized to express transforming growth factor-β_1 and β_3 genes *in vivo*. We have documented widespread cellular gene expression of both isoforms, confirmed in human as well as murine lung. This suggests significant roles for both peptides in normal pulmonary homeostasis. These are likely to include regulation of cellular proliferation, growth and differentiation, immune function and extracellular matrix metabolism. Future studies utilizing this technique should provide further information concerning the role of individual genes during lung development and in the pathogenesis of a wide variety of lung diseases.

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