RESEARCH COMMUNICATION

A novel transforming growth factor β_2 antisense transcript in mammalian lung

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Transforming growth factor (TGF) β_2 gene expression was examined in murine, rat and human lung by *in situ* hybridization with riboprobes. Hybridization signal was observed in a variety of cells with the sense probe, and Northern-blot analysis with

this probe demonstrated the presence of a novel 3.5 kb transcript. This first report suggesting the existence of a natural TGF β_2 antisense transcript raises the possibility that such a transcript may play a role in regulating TGF β_2 production.

INTRODUCTION

The transforming growth factor (TGF) β family consists of at least five 25 kDa homodimeric isoforms, of which TGF β_{1-3} are present in mammalian tissues. These multifunctional peptides are thought to play important roles in lung development [1,2]. TGF β_1 has been implicated in the pathogenesis of lung disease including asthma [3], malignancy [4–6] and a variety of interstitial lung diseases including pulmonary fibrosis [7–9]. The role of the other two mammalian isoforms is not yet clear. During the course of preliminary studies investigating the role of TGF β isoforms in the development of pulmonary fibrosis, TGF β_{1-3} gene expression was localized in normal murine lung.

In situ hybridization studies with isoform-specific digoxigenin-labelled riboprobes showed that TGF β_1 and TGF β_3 mRNA transcripts were abundant in normal murine lung, but hybridization with the TGF β_2 probes yielded unexpected results. Hybridization with the sense probe yielded a positive signal, whereas hybridization with the antisense probe yielded little or no signal. The purpose of the studies described below was to examine the hypothesis that this finding is due to the presence of a natural TGF β_2 antisense transcript in normal mammalian lung.

METHODS AND MATERIALS

Lung tissue and cells

In situ hybridization was performed in murine, rat and human lung. Northern-blot analysis was performed in rat lung and murine mesothelioma cells. Six adult mice and six adult rats were killed by pentobarbitone overdose. For *in situ* hybridization, lungs were fixed by intratracheal instillation of 4% paraformaldehyde at a pressure of 25 cmH₂O (where 1 cmH₂O = 98.2 Pa). The trachea was ligated just caudal to the larynx and the heart and lungs were removed together. After 4 h immersion in fixative, lung tissue was transferred to 15% sucrose in PBS prior to dehydration and embedding in paraffin wax. For studies in human lung, lung tissue was obtained from two patients undergoing resection for carcinoma. Only macroscopically normal lung peripheral to the resection was used for these studies. For

Northern-blot analysis, rat lung tissue was removed, homogenized immediately in TRIzol reagent (Gibco, Paisley, Scotland, U.K.) and frozen at –80 °C prior to RNA extraction. Murine mesothelioma cells (AC29) known to produce TGF β_2 [10] were cultured to confluence in Dulbecco's modified Eagle's medium, containing 200 units/ml penicillin, 200 units/ml streptomycin, 4 mM glutamine, 5 ng/ml epidermal growth factor and supplemented with 5% fetal calf serum. Cell layers were harvested into TRIzol and frozen at –80 °C prior to RNA extraction.

In situ hybridization

Pre-hybridization treatments have already been described [11]. Briefly, 5 μ m tissue sections were placed on silanized slides. After dewaxing, sections were rehydrated through a series of ethanol washes of decreasing concentration, which was followed by immersion in 0.14 M sodium chloride and PBS before refixing in 4% paraformaldehyde. Sections were permeabilized with proteinase K (Life Technologies, Paisley, Scotland, U.K.) at a concentration of 20 μ g/ml in 50 mM Tris/HCl (pH 7.5)/5 mM EDTA for 10 min before refixing with paraformaldehyde. Sections were acetylated by immersion in 0.1 M triethanolamine containing 0.25% acetic anhydride and dehydrated through increasing concentrations of ethanol.

The *in situ* hybridization protocol was as published previously [11]. Hybridization buffer [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 (0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.002% BSA)/500 mg/ml yeast tRNA] was mixed with digoxigenin-labelled riboprobe (prepared as described below) to give a probe concentration of 20 ng/ μ l. 25 ml of hybridization solution was applied to each slide and sections were incubated for 16 h at 50 °C in a sealed chamber humidified with 50% formamide in 2× SSC (0.15 M NaCl/0.015 M sodium citrate). All subsequent incubations were performed at room temperature. Slides were washed in 4× SSC for 30 min, then in 0.2× SSC for 30 min. They were rinsed in Tris base saline [TBS; consisting of 0.1 M Tris (pH 8.0)/0.15 M NaCl] for 5 min, before incubation for 30 min with antibody blocking solution, consisting of 5% BSA and 5% normal sheep serum diluted in TBS with 0.1%

Abbreviations used: TGF, transforming growth factor; SSC, 0.15 M NaCl/0.015 M sodium citrate; TBS, Tris base saline; bFGF, basic fibroblast growth factor.

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Figure 1 *In situ* hybridization in normal lung with $TGF\beta_{1-3}$ riboprobes

Results of *in situ* hybridization of sections of normal murine lung hybridized with $TGF\beta_1$ and $TGF\beta_3$ sense (b,d) and antisense (a,c) probes. Hybridized probe appears as red. Gene expression for $TGF\beta_1$ and $TGF\beta_3$ was widespread in normal lung, being particularly prominent in bronchiolar epithelium (arrowheads in a and c) and alveolar macrophages. Unexpected results were obtained with the $TGF\beta_2$ probes, with positive signal obtained with the sense (f) but not the antisense (e) probe. The arrowheads indicate hybridization signal noted in bronchiolar epithelium, alveolar macrophages and alveolar epithelium. The lower four panels show results of *in situ* hybridization of sections of normal human (g,h) and rat (i,j) lung hybridized with sense (h,j) and antisense (g,i) $TGF\beta_2$ riboprobes. Similar results to those obtained in murine lung were obtained using the $TGF\beta_2$ riboprobes in normal human and rat lung, with the sense probe yielding positive signal and the antisense probe producing little or no signal.

Tween 20^R (Sigma–Aldrich, Poole, Dorset, U.K.). After two further 5 min washes in TBS, sections were incubated with antibody solution for 30 min. This consisted of a 1:100 dilution of anti-digoxigenin alkaline phosphatase Fab fragments (Boehringer Mannheim, U.K.) in 1% BSA in TBS with Tween 20^R. Sections were then washed twice in TBS for 5 min each.

For detection of hybridized probe, sections were incubated with the alkaline phosphatase substrate New Fuschin Red (Dako, U.K.), containing 1 mM levamisole to inhibit endogenous alkaline phosphatase activity. 200 μ l of reagent was applied to each section for 20 min. Slides were then rinsed in distilled water, counterstained with haematoxylin and mounted in glycerol. New Fuschin Red yields a red colour at the site of the hybridized probe. Each experiment *in situ* was repeated at least twice. Sections were examined independently by two of the authors (R. K. Coker and R. J. McAnulty).

Probe preparation

Riboprobes were synthesized from transcript-specific murine $TGF\beta$ cDNA constructs in pGEM vectors ($TGF\beta_1$ and $TGF\beta_3$) and in the SP72 vector ($TGF\beta_2$). Constructs were obtained by deleting the highly conserved regions of the cDNAs [12]. The $TGF\beta_1$ construct included nucleotides 421–1395 of the murine

DNA, and contained 764 bp of the N-terminal glycopeptide (precursor) region and 210 bp of the mature region. The $TGF\beta_2$ construct contained 442 bp of the N-terminal glycopeptide region (1511–1953). The $TGF\beta_3$ construct contained 609 bp of the N-terminal glycopeptide region (831–1440). Digoxigenin-labelled riboprobes were synthesized by transcription *in vitro* from linearized cDNA templates according to the manufacturers' protocol (Boehringer Mannheim). cDNA probes for Northern-blot analysis were labelled with ³²P to a specific activity of approx. 2×10^6 d.p.m./ng by random prime labelling according to the manufacturer's instructions (Amersham, Bucks, U.K.).

Northern-blot analysis

Total RNA was extracted from murine mesothelioma cells (AC29) or from rat lung following homogenization in TRIzol. RNA extraction using TRIzol is based on methods described previously [13] and was performed according to the manufacturer's instructions. Polyadenylated (poly A⁺) RNA was isolated from total RNA with a biotinylated oligo (dT) probe (Promega, Southampton, U.K.). 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 eluted with water. Poly A⁺ RNA

samples (10 μ g) were electrophoresed through a 1 % denaturing agarose-formaldehyde gel and blotted on to a nylon membrane (Hybond N, from Amersham for 32 P-labelled probes, or from Boehringer Mannheim for digoxigenin-labelled probes). RNA was crosslinked by brief exposure to UV light, using a UV transilluminator, followed by baking at 80 °C for 2 h.

Hybridization was performed overnight at 42 °C in 50% formamide/5 × [0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA]/5 × Denhardt's reagent/0.1% SDS/100 mg/ml denatured salmon sperm DNA. Post-hybridization washes were performed twice in 2 × SSC/0.1% SDS for 10 min at 42 °C, and then in 1 × SSC/0.1% SDS for 15 min at 42 °C. Hybridized ³²P-labelled probes were detected by phosphorimaging with a Fuji Bas system 1000 (Raytek Scientific Ltd, Sheffield, U.K.). Hybridized digoxigenin-labelled probes were detected by chemiluminescence assay (Boehringer Mannheim). Phosphorimaging records and autoradiographs were scanned by laser densitometry (Image Master 1D, Pharmacia, Milton Keynes, U.K.).

Characterization of TGF β_2 probes

TGF β_2 probe orientation in the vector was confirmed in two ways. Asymmetric restriction enzyme digests were performed with *Clal* and resulting fragments examined by electrophoresis in 1% agarose gel. Dideoxy-mediated chain-termination sequencing was performed with T7 polymerase (United States Biochemical Sequenase Version 2.0, Amersham). Digoxigenin incorporation in TGF β_2 sense and antisense probes was assessed by chemiluminescence assay (Boehringer Mannheim).

RESULTS

In situ hybridization

Figure 1 shows results of *in situ* hybridization of sections of normal murine lung hybridized with the TGF β riboprobes. Panels a–d show sections hybridized with the antisense (a,c) and sense (b,d) TGF β_1 and TGF β_3 probes, respectively. The remaining panels demonstrate results of *in situ* hybridization of sections of normal murine (e,f), human (g,h) and rat (i,j) lung hybridized with sense (f,h,j) and antisense (e,g,i) TGF β_2 riboprobes. TGF β_1 and TGF β_3 antisense probes yielded hybridization signal (a,c) whereas little or no signal was apparent using the sense probes (b,d). TGF β_1 and TGF β_3 mRNA transcripts were widespread in normal lung, being particularly evident in bronchial epithelium and alveolar macrophages.

Unexpected results were obtained using the TGF β_2 riboprobes. Hybridization with the TGF β_2 sense probe (f) in murine lung yielded positive signal consistently, whereas hybridization with the antisense probe (e) yielded little or no signal. These results were obtained on two separate occasions and with two different

Figure 3 Northern-blot analysis with radiolabelled cDNA $TGF\beta_{1-3}$ probes

Results of Northern-blot analysis of poly A⁺ RNA from AC29 cells with each of the radiolabelled TGF β cDNA probes. Size markers (kb) are shown on the left. The following transcripts were seen: TGF β_1 , 2.3 kb; TGF β_2 , multiple transcripts from 3.7 to 6.7 kb; TGF β_3 , 3.5 kb, confirming the specificity of the probes for each of the three TGF β isoforms.

batches of freshly prepared probe. Similar results were obtained using the TGF β_2 riboprobes in normal human (g,h) and rat (i,j) lung. The distribution of signal obtained with the TGF β_2 sense probe was similar to that observed for TGF β_1 and TGF β_3 , being predominant in bronchiolar epithelium and alveolar macrophages.

Characterization of TGF β_2 probe

Asymmetric restriction-enzyme digests confirmed correct orientation of the probe in the vector, as did sequence analysis. Figure 2 shows the sequence homology obtained between the TGF β_2 sense probe and mRNA for TGF β_2 mRNA for murine TGF β_2 , using data derived from the FASTA database searching program (Human Genome Mapping Project CRC, Cambridge, UK). The percentage identity was 86.3 and no other sequence showed greater similarity.

The ratio of digoxigenin labelling of the TGF β_2 antisense probe compared with the sense probe was 1.2:1, thereby indicating that the positive signal obtained with the sense probe was not due to unequal digoxigenin incorporation in the probes.

Northern-blot analysis

Figure 3 shows results of Northern-blot analysis of poly A⁺ RNA from murine mesothelioma AC29 cells with each of the radiolabelled TGF β cDNA probes. Size markers (in kb) are

1582

TCTACAGACCCTACTTCAGAATCGTCCGCTTTGATGTCTCAACAATGGAGAAAAATGC TT CGAATCTGGTGAAAGCAGAGTTCA
| ||||| | ||| | || ||||||||| ||||| | || || || ||
TTTACAGACCTTACTT.AGA.....TGR TTTGATGTCTCAACAATGGAGAAAGAR....RTGATTCTGGTGAAAGGAAGAGTTCA

4

1665

79

Figure 2 Sequencing of the TGF β_2 probe

The sequence homology obtained between the TGF β_2 sense probe and mRNA for murine TGF β_2 , using data derived from the FASTA database searching program. Percentage identity was 86.3.

Figure 4 Northern-blot analysis with digoxigenin-labelled TGF β riboprobes

(a) Shows results of Northern-blot analysis of poly A⁺ RNA from rat lung using digoxigenin-labelled TGF β_2 antisense (lane 1) or sense (lane 2) riboprobes. The antisense probe detected four transcripts of 4, 5, 5.9 and 6.9 kb. The sense probe detected one 3.5 kb transcript, the absorbance of which was twice that of the other four transcripts combined. Similar results were obtained with poly A⁺ RNA from AC29 cells (b). The antisense probe detected four transcripts of 3.7, 4.8, 5.8 and 6.7 kb and the sense probe detected a single prominent 3.6 kb transcript.

shown on the left. The following transcripts were seen: TGF β_1 , 2.3 kb; TGF β_2 , multiple transcripts from 3.7 to 6.7 kb; TGF β_3 , 3.5 kb, confirming the specificity of the probes for each of the three TGF β isoforms.

Figure 4(a) shows results of Northern-blot analysis of poly A⁺ RNA from rat lung using digoxigenin-labelled TGF β_2 antisense (lane 1) or sense (lane 2) riboprobes. The antisense probe detected four transcripts of 4, 5, 5.9 and 6.9 kb. The sense probe detected one 3.5 kb transcript, the absorbance of which was twice that of the other four transcripts combined. Similar results were obtained with poly A⁺ RNA from murine mesothelioma cells (AC29) and are shown in Figure 4(b). The antisense probe detected four transcripts of 3.7, 4.8, 5.8 and 6.7 kb, and the sense probe detected a single prominent 3.6 kb transcript.

DISCUSSION

The results of *in situ* hybridization with the TGF β_2 probes were unexpected and contrasted with those obtained using the TGF β_1 and TGF β_3 probes. There were four possible explanations for these findings. 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. Secondly, the sense probe could be hybridizing with an unrelated species. This is unlikely given the sequencing results. Thirdly, the sense probe could be more highly labelled with digoxigenin than the antisense probe. This was excluded using a chemiluminescence assay to assess labelling. Finally, a naturally occurring TGF β_2 antisense transcript may be present in lung tissue. The results of Northern-blot analysis using

TGF β_2 riboprobes in rat lung and murine mesothelioma cells would be consistent with this hypothesis. Taken together, the *in situ* hybridization and Northern-blot-analysis data suggest that a natural TGF β_2 antisense transcript is present in murine, rat and human lung. Its expression across different mammalian species suggests that it may play a significant role in TGF β_2 biology.

The possibility of an endogenous TGF β_2 antisense RNA is potentially very important because, in prokaryotes, endogenous antisense RNAs have been shown to bind to a complementary region of a target RNA and affect its function [14]. Regulation by antisense RNA was first discovered while studying replication of the *Escherichia coli* plasmid ColE1. Plasmid replication is dependent on the formation of an RNA primer whose precursor RNA is functional only when it adopts a certain structure during its synthesis. Interaction of a small antisense RNA to the primer precursor inhibits formation of this structure, and consequently prevents plasmid replication [15]. Natural antisense RNAs may be transcribed from the complementary DNA strand [16,17] and new examples of regulation by natural antisense transcripts are being described [18,19].

Evidence is now accumulating for naturally occurring antisense RNA regulation in eukaryotes [20]. There are several reports in eukaryotes of both strands of a DNA segment being transcribed, or of complementary RNAs being detected. Examples in vertebrates include complementary sequences to chicken myosin heavy chain [21] and murine dihydrofolate reductase [22]. The former can inhibit translation of myosin heavy-chain mRNA *in vitro*.

Antisense transcripts for cytokines have also been described. An antisense RNA transcript for the basic fibroblast growth factor (bFGF) gene has been identified and characterized [23,24]. bFGF is overexpressed in human glioma cells and increased mRNA stability has been shown to contribute to elevated bFGF mRNA levels in these cells. The authors found that normal rat tissues and human breast cancer cells contained low levels of bFGF mRNA, but high levels of the antisense RNA molecule. In contrast, the antisense transcript was undetectable in tissues overexpressing bFGF mRNA. The reciprocal relationship between bFGF mRNA and antisense expression supports a role for the antisense transcript in regulating bFGF gene expression.

An endogenous TGF β antisense also has a precedent, since a naturally occurring antisense RNA to TGF β_3 mRNA has been reported in chick heart [25]. In this study, spatial and temporal analysis revealed that TGF β_3 mRNA is concentrated in the atrioventricular canal tissue where valve formation occurs. The antisense transcript is also expressed in the atrioventricular canal. Expression of the antisense transcript increased during the period of development examined. The increase coincided with the loss of capacity of the atrioventricular canal myocardium to induce the epithelial-mesenchymal transformation of endothelial cells, which initiates valve formation. The temporal expression of the antisense transcript during this stage of development suggests that it may play a role in the regulation of TGF β_3 production during cardiac-valve formation. At present there are no published data on antisense transcripts in the lung or data showing that TGF β antisense transcripts are playing roles in regulating matrix production *in vivo*.

We feel that this observation merits urgent consideration. If such a transcript were identified conclusively by cloning, it would have important implications for studies of TGF β_2 gene expression and for those of other cytokines. The existence of antisense transcripts is unlikely to be an unusual phenomenon, merely one that has not hitherto been recognized widely in mammals. Rigorous controls for *in situ* hybridization studies using sense and antisense riboprobes would have to be included wherever possible. Since Pelton and colleagues [12] were able to dem-

onstrate murine TGF β_2 transcripts using identical TGF β_2 riboprobes to those we employed, the appearance of a natural antisense may be strain- or age-specific.

If expression of the TGF β_2 antisense transcript were confirmed in normal and diseased lung, it would have important implications for our understanding of the regulation of TGF β gene expression *in vivo*. Such an antisense transcript may be regulating TGF β_2 gene expression in a variety of ways, as outlined above. An endogenous regulatory mechanism of this kind would have exciting implications for future therapies directed at modifying TGF β function in a variety of diseases where TGF β is implicated.

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REFERENCES

- 1 Pelton, R. W., Saxena, B., Jones, M., Moses, H. L. and Gold, L. I. (1991) *J. Cell Biol.* **115**, 1091–1105
- 2 Schmid, P., Cox, D., Bilbe, G., Maier, R. and McMaster, G. K. (1991) *Development* **111**, 117–130
- 3 Robinson, D. S., Durham, S. R. and Kay, A. B. (1993) *Thorax* **48**, 845–853
- 4 Bergh, J. (1988) *Am. J. Pathol.* **133**, 434–439
- 5 Fynan, T. M. and Reiss, M. (1993) *Crit. Rev. Oncog.* **4**, 493–540
- 6 Maeda, J., Ueki, N., Ohkawa, T., Iwahashi, N., Nakano, T., Hada, T. and Higashino, K. (1994) *Clin. Exp. Immunol.* **98**, 319–322
- 7 Broekelmann, T. J., Limper, A. H., Colby, T. V. and McDonald, J. A. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6642–6646
- 8 Corrin, B., Butcher, D., McAnulty, R. J., du Bois, R. M., Black, C. M., Laurent, G. J. and Harrison, N. K. (1994) *Histopathology* **24**, 145–150
- 9 Khalil, N. and O'Connor, R. N. (1995) in *Pulmonary Fibrosis* (Phan, S. H. and Thrall, R. S., eds.), pp. 627–645, Marcel Dekker, New York
- 10 Fitzpatrick, D. R. (1994) *Growth Factors* **11**, 29–44
- 11 Coker, R. K., Laurent, G. J., Shahzeidi, S., Hernandez-Rodriguez, N. A., Pantelidis, P., du Bois, R. M., Jeffery, P. K. and McAnulty, R. J. (1996) *Eur. Resp. J.* **9**, 2501–2507
- 12 Pelton, R. W., Johnson, M. D., Perkett, E. A., Gold, L. I. and Moses, H. L. (1991) *Am. J. Respir. Cell Mol. Biol.* **5**, 522–530
- 13 Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- 14 Eguchi, Y., Itoh, T. and Tomizawa, J. (1991) *Annu. Rev. Biochem.* **60**, 631–652
- 15 Masukata, H. and Tomizawa, J. (1986) *Cell* **44**, 125–136
- 16 Cock, J. M., Swarup, R. and Dumas, C. (1997) *Mol. Gen. Genet.* **255**, 514–524
- 17 Sureau, A., Soret, J., Guyon, C., Gaillard, C., Dumon, S., Keller, M., Crisanti, P. and Perbal, B. (1997) *Nucl. Acids Res.* **25**, 4513–4522
- 18 Tagieva, N. E. and Vaquero, C. (1997) *J. Gen. Virol.* **78**, 2503–2511
- 19 Gerdes, K., Gulyaev, A. P., Franch, T., Pedersen, K. and Mikkelsen, N. D. (1997) *Annu. Rev. Genet.* **31**, 1–31
- 20 Knee, R. and Murphy, P. R. (1997) *Neurochem. Int.* **31**, 379–392
- 21 Heywood, S. M. (1986) *Nucleic Acids Res.* **14**, 6771–6772
- 22 Farnham, P. J., Abrams, J. M. and Schimke, R. T. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3978–3982
- 23 Murphy, P. R. and Knee, R. S. (1994) *Mol. Endocrinol.* **8**, 852–859
- 24 Li, A. W., Seyoum, G., Shiu, R. P. and Murphy, P. R. (1996) *Mol. Cell. Endocrinol.* **118**, 113–123
- 25 Potts, J. D., Vincent, E. B., Runyan, R. B. and Weeks, D. L. (1992) *Dev. Dyn.* **193**, 340–345