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The 3’ untranslated region of tumor necrosis factor-a is highly conserved in idiopathic pulmonary fibrosis (IPF).

Abstract

Tumour necrosis factor alpha (TNF-a), a pro-inflammatory cytokine essential for the function of the immune system, has been implicated in the pathogenesis of idiopathic pulmonary fibrosis (IPF). Production of TNF-a is regulated at both the transcriptional and post-transcriptional levels by a number of factors including interleukin-10 (IL-10). We have shown that there is significant TNF-a activity in patients with IPF, despite the presence of significant amounts of IL-10 and Il-10R. IL-10 is thought to influence the tight translational repression of TNF-a mRNA in

pulmonary macrophages. The essential element in this regulation is the AU-rich element (ARE) present in the 3' untranslated region of TNF-a. We hypothesised that polymorphism in the 3' UTR region of TNF-a explains the apparent failure of IL-10 to down regulate TNF-a in patients with IPF. Using single strand conformation polymorphism (SSCP) analysis, we have screened this region in 96 patients with IPF, using nine sets of overlapping PCR primers. All samples were successfully amplified for each primer set, but SSCP analysis was unable to detect point mutations or polymorphisms in the patients in any of the nine fragments. Results from this study suggest that the 3' UTR region of TNF-a is highly conserved in IPF and mutation of this region is unlikely to be involved in the pathogenesis of IPF.

Full text

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive disorder characterised by accumulation of inflammatory cells in the airspace and fibrosis of the lung parenchyma. The cause of IPF remains unclear, but it is thought that chronic inflammation contributes to the pathological process. The inflammatory response is an essential component of host defence that is tightly regulated *in vivo*. In the lung, alveolar macrophages (AM) participate in the host immune response, at least in part, through production of cytokines and chemokines initiated in response to an array of extracellular stimuli [1-3]. One such pro-inflammatory cytokine, tumour necrosis factor-alpha (TNF-alpha), has been implicated in the pathogenesis of IPF.

TNF-alpha not only enhances adherence of inflammatory cells to the endothelium and their migration into alveolar inflammatory sites, but also activates the production of other inflammatory mediators by monocytes and neutrophils [4]. As the inflammatory process may generate significant tissue injury, it is homeostatically controlled by inhibitory mechanisms through the action of anti-inflammatory cytokines such as interleukin-10 (IL-10) [3].

Many studies have already investigated polymorphisms in the TNF-alpha gene. Although conservative mutations do not affect the amino acid sequence, they may influence protein expression in a variety of ways such as altering mRNA splicing, mRNA stability and levels of gene transcription. Polymorphisms within

the 5'- regulatory region may have a significant effect on transcription, since they may alter transcription factor binding sites within gene promoters or the structure of enhancers and silencers at more remote regulatory sites [5]. In several studies, associations between specific TNF-alpha polymorphisms and clinical outcome of the disease have been investigated. Association with TNF-alpha promoter region polymorphisms has been shown in a wide range of disorders including asthma, multiple sclerosis and chronic lymphocytic leukaemia. Similarly, several of the TNF-alpha promoter polymorphisms have been shown to influence the expression of TNF-alpha protein, with increased expression reported with polymorphism at -1031, -862, -308 and -238 upstream of the translational start codon [6].

However, the production of TNF-alpha is largely controlled post-transcriptionally at the level of mRNA stability and translational efficacy [7-9]. Extensive studies have revealed that the 3' untranslated region (3' UTR) of TNF-alpha mRNA contains sequences that suppress the translation of TNF-alpha in non-activated macrophages and other cell types [7-9]. Following stimulation by agents such as gamma interferon (IFN-gamma), or IL-1, the TNF-alpha mRNA remains poorly translated in macrophages and, consequently, the amount of TNF-alpha produced remains low. However, certain stimuli, such as LPS, induce transcription of TNF-alpha and abolish translational repression, allowing efficient production of TNF-alpha [10]. The TNF-alpha mRNA 3' UTR contains multiple repeats of an AUUU motif (AU-rich element, ARE) common to various cytokine and proto-oncogene mRNAs [11-13]. AU-rich elements of various genes are known to be recognition sequences of several RNA binding proteins [14-18]. There is abundant evidence indicating that AREs found in the 3' UTR of short-lived mRNAs are important in determining mRNA stability and translation [19, 20]. In a previous work using primary mouse macrophages, it was observed that IL-10 suppressed TNF-alpha gene expression through selective destabilisation of mRNA [21]. Further study defined the ARE motif in the 3' UTR as both necessary and sufficient for IL-10-mediated destabilisation of reporter mRNA [21].

Although there have been numerous studies investigating polymorphism of the TNF-alpha promoter, investigations of the 3' UTR of cytokine genes are limited in number. In this study, we hypothesised that polymorphism in the 3' UTR region of TNF-alpha would explain our observations of TNF-alpha resistance to IL-10 in IPF. To investigate this, we screened the final exon of the TNF-alpha gene, which

includes the translational stop codon, the poly-A signal sequence and several ARE, for mutation using single strand conformation polymorphism (SSCP) analysis. A series of nine overlapping polymerase chain reaction (PCR) primers were designed and synthesised to cover the entire exon and were used to screen DNA from 92 patients with IPF and 30 normal controls.

MATERIALS AND METHODS

Subjects. All patients with IPF were diagnosed according to results of a CT scan and transbronchial biopsy performed at presentation of the illness. The IPF group consisted of 92 patients (61 male, 31 female) with a mean age of 66 years (range 37-86), 65% of whom were smokers or ex-smokers. These patients had CT-scan appearances and biopsies compatible with the usual interstitial pneumonia (UIP) group of IPF [22]. The normal control population of 30 individuals (18 male/12 female) consisted of healthy volunteers and patients undergoing bronchoscopy with normal lung function, in whom no abnormality was detected (*e.g.* unexplained haemoptysis, persistent cough). The normal controls had a mean age of 32.5 years (range 20-62), 25% of whom were smokers or ex-smokers.

DNA extraction. A standard high salt DNA extraction method was used [23]. Briefly, 5 ml of peripheral blood were collected in EDTA tubes and centrifuged for 10 min at 1,300 x G. The mononuclear cell layer was transferred to a conical tube and 4 ml red cell lysis buffer (0.144 M NH_4Cl, 1 mM $NaHCO_3$) added. After 20 min the tube was spun for 10 min at 1,300 x G. Red cell lysate was discarded and the white cell pellet resuspended in 1.5 ml nuclei lysis buffer (10 mM TRIS-HCL, pH 8.2, 0.4 NaCl, 2 mM EDTA pH 8.0). 0.3 ml 1 x proteinase K solution (2 mg/ml proteinase K, 1% W/V SDS, 2 mMEDTA pH 8.0) and 0.2 ml 10% W/V SDS were added, mixed and incubated for 18 hours at 37° C. 0.5 ml of 6 M NaCl was added and mixed for 15 seconds before being centrifuged for 25 min at 1,300 x G. The supernatant was added to 4 ml of absolute ethanol in a clean tube. The precipitated DNA was removed, resuspended in sterile water and stored at 4° C.

Single strand conformation polymorphism analysis (SSCP). This method exploits the fact that nucleotide mutations change the conformation of single stranded DNA and results in altered mobilities of heat-denatured PCR products in non-denaturing polyacrylamide gels. PCR primers were designed which gave overlapping products of approximately 200-300 bp in length, to cover the entire

3' untranslated region of TNF-alpha (Table 1). PCR mixes (25 mul) containing 5 pmol each of forward and reverse primers, 2.5 mM $MgCl_2$, 200 muM of each dNTP, 1 x Taq buffer (75 mM Tris-HCL pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% V/V Tween), 0.3 units of AS Gold (Hybaid, Middlesex, UK) were added to 200 ng patient genomic DNA. Following an initial denaturation at 94° C for 1 min, 35 cycles of 94° C for 30 seconds, 60° C for 30 seconds and 72° C for 30 seconds were performed followed by a final extension at 72° C for 5 min. An equal volume of denaturing solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to each amplicon and the samples denatured at 95° C for 5 min and placed on ice immediately. Ten mul were loaded on MDE high resolution polyacrylamide (FMC, Maine, USA) in a triple wide minigel system with 1 x TBE. To maximise polymorphic detection efficiency three gel conditions were used A) 0.5% MDE, 4° C, 225 V for 120 min, B) 0.75% MDE, room temperature, 175 V 180 min, C) 0.5% MDE with 5% glycerol, room temperature, 150 V for 180 min. After electrophoresis, the gel was stained in 1 x SYBR Gold (Molecular probes, Oregon, USA) and visualised on a Dark Reader transilluminator (Clare Chemical Research, Ross-on Wye, UK).

RESULTS

Conditions for the screening of the 3' UTR by SSCP were optimised using samples which had previously been genotyped for a polymorphism in the TNF-alpha gene promoter region. The polymorphism lies 238 base pairs (-238) upstream of the translation start codon of TNF-alpha and is one of several well characterised polymorphisms in this region. Five samples which had previously been genotyped for the -238 polymorphism using heteroduplex analysis were screened. Both homozygotes and heterozygotes were clearly distinguishable under all gel conditions used in this study (Figure 1) and demonstrate the viability of SSCP under these conditions for polymorphism detection.

All 92 patients with IPF and all 30 normal controls were successfully screened for the 3' UTR of TNF-alpha using SSCP. The nine PCR primer sets used in this study covered a region of 1,607 nucleotides which included the region which codes for the entire final exon of the TNF-alpha mRNA sequence (Figure 2). This exon contains the translational stop codon, the poly-A signal sequence and several ARE which have been shown to be highly involved in TNF-alpha translational control and hence TNF-alpha production. All samples were successfully amplified for each primer set, indicating that large scale deletion or re-

arrangement of the 3' UTR is unlikely to be involved in TNF-alpha dysregulation in IPF. Similarly, SSCP analysis was unable to detect point mutations or polymorphisms in the patients or normal controls in any of the nine fragments amplified. A representative gel is shown in Figure 3.

DISCUSSION

The fundamental role of TNF-alpha in inflammation has led to interest in the regulatory mechanisms controlling its production and activity, that may have a significant role to play in the modulation of diseases such as IPF. IL-10 is one cytokine that has been studied in this context. Mechanisms of IL-10 suppressive activity, although incompletely characterised, appear to depend on the gene of interest, the nature of the stimulus and the cell type. Various studies have demonstrated that IL-10 can reduce the level of gene transcription, decrease the stability of mRNAs and reduce their translation [24-27]. TNF-alpha has been shown to stimulate IL-10 production by human monocytes and macrophages, suggesting a normal endogenous feedback system for control of immune responses and inflammation [28, 29]. In a previous study, we investigated the relationship between IL-10, TNF-alpha and their receptors in AMs in IPF and showed significantly greater constitutive and LPS-induced production of bioactive TNF-alpha in IPF compared to normal controls [30]. However, this increase was not seen at the mRNA level. It was shown using a quantitative RT-PCR method, that there was no significant difference between groups in constitutive TNF-alpha mRNA expression and only a slight increase in overall levels after LPS stimulation. The elevated levels of TNF-alpha were seen in AM despite the increased presence of the natural inhibitors to TNF-alpha, IL-10 and soluble CD120b (TNF-RII). These data suggested that normal homeostatic mechanisms which regulate AM derived TNF-alpha are ineffective in IPF.

Although we have shown increased levels of TNF-alpha protein in IPF, no difference in the level of TNF-alpha mRNA was found between groups. The role of transcription and translation is complicated. Some studies suggest transcription is the main control mechanism of TNF-alpha production [31], while others have shown that response to several viral challenges results, to a great extent, from TNF-alpha mRNA translational activation [32]. In resting macrophages, TNF-alpha synthesis is low because TNF-alpha transcription is weak and TNF-alpha mRNA translation is severely repressed. The translational repression is mediated by the AU-rich sequence in the TNF-alpha mRNA 3' UTR

region. Macrophage activation by LPS results in NF-kB-dependent activation of TNF-alpha gene transcription, depression of TNF-alpha mRNA translation and secretion of TNF-alpha protein. In mouse macrophage cell lines, IL-4 and IL-13 inhibit LPS-induced TNF-alpha production mainly at the translational level, mediated by the UA-rich sequence present in its 3' UTR region [33]. Similarly, IL-10 has been shown to suppress TNF-alpha gene expression in mouse macrophages through selective destabilisation of mRNA probably through AU-rich elements in the 3' untranslated region which have been shown to be necessary and sufficient for IL-10-mediated destabilisation of mRNA [31]. Therefore, lack of normal 3' UTR function may allow greater TNF-alpha mRNA stability and/or reduce translational repression, allowing increased TNF-alpha production without increased transcriptional activity.

To investigate the possibility of abnormal 3' UTR function affecting TNF-alpha translation and/or mRNA stabilisation we screened the 3' UTR region of TNF-alpha for mutation using SSCP analysis. The 92 patients with IPF and 30 normal controls were screened for the 3' UTR of TNF-alpha using SSCP. The nine PCR primer sets used in this study covered a region of 1,607 nucleotides which include the translational stop codon, the poly-A signal sequence and several AREs. All samples were successfully amplified for each primer set, indicating that large scale deletion or rearrangement of the 3' UTR is unlikely to be involved in TNF-alpha disregulation in IPF. Similarly, SSCP analysis was unable to detect point mutations or polymorphisms in the patients or normal controls in any of the nine fragments amplified. The viability of SSCP as a technique to look for novel polymorphisms has been demonstrated to good effect in several studies on cytokines and receptors [34, 35]. Also, the conditions used in this study were able to successfully detect known polymorphisms in previously genotyped samples, and have subsequently been used in our laboratory to successfully detect novel polymorphisms in the IL-10 gene (Freeburn, *et al.*, unpublished). Therefore, the failure to detect any polymorphism in the 3' UTR of TNF-alpha is unlikely to be due to technical problems and suggests that this region is highly conserved in both the IPF patients and normal controls. Although results from this study suggest that polymorphisms of the 3' UTR region of TNF-alpha is uncommon and is unlikely to be involved in the pathogenesis of IPF, it does not rule out the possibility that proteins which bind in this region could be involved.

At least two putative protein binding regions have been identified in the area investigated in this study. In mice, deleting these TNF AU-rich elements (ARE)

affected mechanisms responsible for TNF mRNA destabilisation and translational repression in hemopoietic and stromal cells. In stimulated conditions, TNF ARE were required for both the alleviation and reinforcement of message destabilisation and translational silencing [36]. Moreover, a recent study using immunopeptides has demonstrated that in addition to AREs other nucleotide sequences in the 3' UTR region of TNF-alpha mRNA are involved in translational control of TNF-alpha expression [37]. Comparison of the nucleotide sequence of TNF-alpha 3' UTRs from various species reveals that both the sequence and the position of the two putative protein binding regions are highly conserved [10]. The importance of this region in the control of TNF-alpha production is confirmed by the cross-species conservation of the nucleotide sequence and suggests that polymorphism in this region could affect TNF-alpha production. However, this study suggests polymorphism of this region is rare and unlikely to be involved in the pathogenesis of IPF.

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Figures

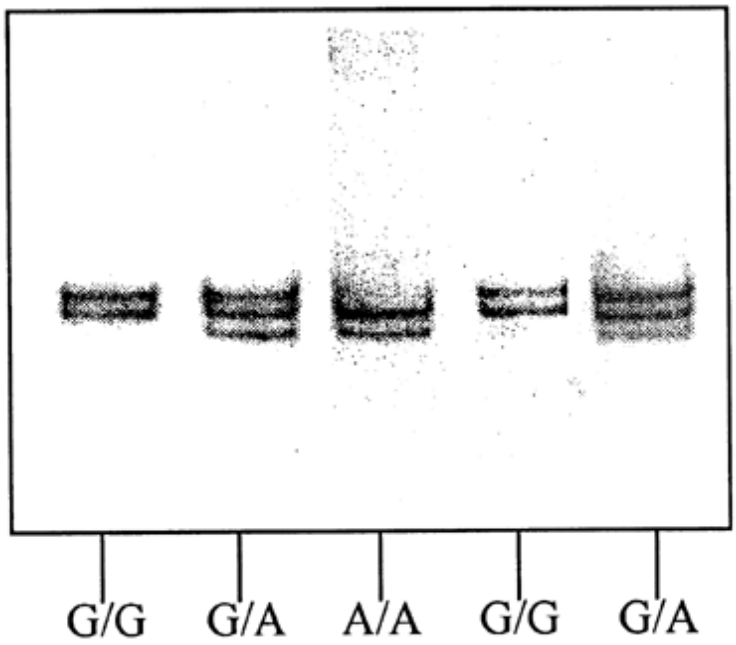


Figure 1

Optimization of SSCP conditions.

Gel conditions for SSCP analysis were optimized using known genotypes. Five samples which had previously been genotyped for the -238 TNF-alpha promoter polymorphism using heteroduplex analysis were used. Homozygotes and heterozygotes were clearly distinguishable under all gel conditions.

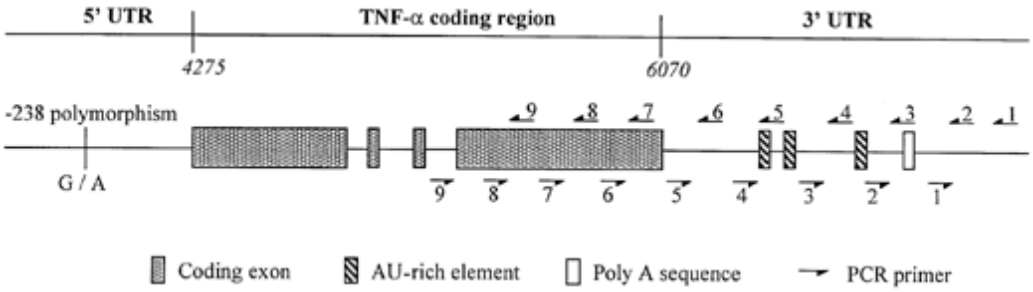


Figure 2

TNF-alpha gene.

Diagram representing the TNF-alpha gene structure with positions of coding exons, AU rich elements and poly A consensus sequence shown. The positions of the PCR primers used to screen the gene using SSCP analysis are also shown.



Figure 3

SSCP analysis of IPF patients in TNF-alpha fragment 7.

MDE polyacrylamide gel (0.75%) showing all patients have normal SSCP pattern for fragment 7.

Table 1
TNF-alpha SSCP PCR primers

	Forward (5' to 3')	Reverse (5' to 3')	Nucleotides amplified *	size bp
1	TGCATCTGCCTCTTCTTGTGG	GAGTTGGAAATTCCCATGCCC	6,896-7,103	208
2	ATAGGCTGTTCCCATGTAGCC	AGGACTTAGGGAGCTTCTTCC	6,666-6,940	275
3	AGCCAGCTCCCTCTATTTATG	ACAGTTGGTCACCAAATCAGC	6,499-6,776	278
4	CTTTGGTTCTGGCCAGAATGC	CCCGGTCTCCCAAATAAATAC	6,366-6,595	230
5	AGCAACAAGACCACCACTCG	AAGGCCTAAGGTCCACTTGTG	6,210-6,441	232
6	GGTCTTACTTTGGGATCATTGC	TAGTGGTTGCCAGCACTTCAC	6,043-6,284	242
7	AACCTCCTCTCTGCCATCAAG	GGGTAATAAAGGGATTGGGGC	5,870-6,128	260
8	CAAACCCTCAAGCTGAGGGG	CCAGATAGATGGGCTCATACC	5,649-5,957	309
9	GATACTCAGAACGTCATGGCC	ACCAGCTGGTTATCTCTCAGC	5,496-5,742	247

* Nucleotides numbered according to Nedospasov S A, *et al.* 1986. Cold Spring Harb. *Symp. Quant. Biol.* 51: 611. GenBank accession-M16441, NID-g339739.

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TNF-a, idiopathic pulmonary fibrosis, polymorphism.

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TNF, cytokine, LPS.

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MUC5AC, MUC5B, mucus-secreting cell line HT29-MTX.

A Jurkat T cell variant resistant to death receptor-induced apoptosis. Correlation with heat shock protein 27 and 70 levels.

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apoptosis, caspases, Hsp27, Hsp70, T lymphocytes.

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MRC5, fibroblasts, TNF receptor, RT-PCR, binding, flow cytometry.

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cytokines, lipocalin, IL-9, 24P3.

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IL-10, recombinant, rat, cytokines.

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