Concise Report

Transforming growth factor beta-1 and gene polymorphisms in oriental ankylosing spondylitis


Objectives. To study serum levels of transforming growth factor beta-1 (TGFβ1) and the expression of TGFβ1 in in vitro peripheral blood mononuclear cell (PBMC) cultures in oriental ankylosing spondylitis (AS) patients, and to determine their association with codon 10 and 25 TGFβ1 gene polymorphisms.

Methods. Serum levels of TGFβ1 were measured by enzyme-linked immunosorbent assay (ELISA). The ability of PBMCs to synthesize TGFβ1 and other cytokines was assessed by in vitro cultures stimulated with mitogen. Genomic DNA was extracted from PBMCs of AS patients (n = 72) or unrelated healthy controls (n = 96). The codon 10 and 25 polymorphisms in the TGFβ1 gene were analysed using standard polymerase chain reaction-based methods.

Results. AS patients had significantly higher serum TGFβ1 levels than controls (P < 0.001). There was no difference in the distribution of codon 10 and 25 TGFβ1 genotypes between AS patients and controls. Incubation of AS and control PBMC with phytohaemagglutinin (PHA) led to upregulation of TGFβ1, interleukin-10, tumour necrosis factor-alpha (TNFα) and interferon-γ (IFNγ) assessed by ELISA. Importantly, PHA-induced TGFβ1 production was significantly enhanced in AS patients compared with normal controls whereas the production of the pro-inflammatory cytokines TNFα and IFNγ was reduced.

Conclusions. Our results show that AS patients express significantly higher levels of serum TGFβ1 independent of the codon 10 and 25 genotype. Activation of AS PBMCs led to enhanced TGFβ1 production accompanied by reduction of TNFα and IFNγ while the converse was observed in normal controls.

Key words: Ankylosing spondylitis, Transforming growth factor beta-1, Cytokines, Polymorphisms.

Ankylosing spondylitis (AS) is characterized by axial skeletal involvement beginning with inflammation in the sacroiliac joints. The majority of patients are histocompatibility antigen HLA-B27 positive, supporting the hypothesis that disease might initially be triggered by bacterial infections that provoke a subsequent T-cell response. Importantly, both CD4+ and CD8+ T cells can be located in sacroiliac joints and entheselial structures in these patients [1, 2]. Transforming growth factor beta-1 (TGFβ1) plays a critical role in the balance of inflammatory processes and has been linked to extracellular matrix synthesis, bone remodelling and fibrosis in AS [3].

TGFβ1 is a pleiotropic cytokine belonging to a family of dimeric polypeptide growth factors that includes bone morphogenic proteins and activins. It regulates embryonic development, the proliferation and differentiation of cells, apoptosis, angiogenesis and wound healing [4, 5]. The expression of TGFβ1 and its receptors is widespread, having been detected in both innate and specific immune response cells as well as on non-immune cells. Locally, TGFβ1 has been shown to have pro-inflammatory properties, whereas systemically it has an immunosuppressive effect [4]. Altered levels of TGFβ1 have been linked to numerous disease states including atherosclerosis and fibrotic disease of the kidneys, liver and lungs. TGFβ1 is important for bone development and fracture healing, and has been shown to cause excessive proliferation of fibroblasts in mice with progressive ankylosis [5, 6].

There are comparatively few studies of cytokines in AS and information on serum levels and expression of TGF in peripheral blood mononuclear cells (PBMCs) in this disease is rather limited. Thus the purpose of our study was to determine protein expression of TGFβ1 both in serum and in in vitro assays and their association with codon 10 and 25 TGFβ1 gene polymorphisms in our population of southern Chinese AS patients.

Patients and methods

Patients and controls

Seventy-two southern Chinese patients who attended the Tan Tock Seng Hospital Department of Rheumatology, Allergy and Immunology in Singapore were recruited in this study which was approved by our hospital Research Ethics committee. All cases had primary AS that met the modified New York criteria for AS [7];
Preparation of PBMCs and in vitro stimulation assays

PBMCs from AS patients and healthy donors were isolated by gradient centrifugation over Histopaque-1077 (Sigma, St Louis, MO) according to the manufacturer’s recommendations and resuspended in complete RPMI 1640 medium supplemented with 2 mM l-glutamine, 100 IU penicillin and 100 mg/ml streptomycin (all obtained from Invitrogen Gibco, Carlsbad, CA). PBMCs (2 x 10⁶/well) were placed in triplicate in 96-well flat-bottom plates (Nunc, Roskilde, Denmark) in complete RPMI 1640 medium supplemented with 5% fetal calf serum (FCS) (Invitrogen Gibco) at 37°C 5% CO₂ and stimulated with phytohaemagglutinin (PHA, Sigma) at 10 µg/ml for 3 days. Culture supernatants were harvested and stored at −20°C until estimated by ELISA.

ELISA

TGFβ1, tumour necrosis factor-alpha (TNFα), interferon-γ (IFNγ) and interleukin-10 (IL-10) levels in culture supernatants were detected using paired antibodies from OptEIA systems (BD Biosciences, San Diego, CA) according to the manufacturer’s instructions. The detection limit was: for TNFα, IFNγ and IL-10 <10 pg/ml; for TGFβ1 <20 pg/ml. Latent TGFβ1 in supernatants was activated by 1 N HCl for 10 min followed by neutralization (1.2 N NaOH/0.5 M HEPES). Given the significant levels of latent TGFβ1 found in bovine serum, background levels in cell-free RPMI 1640 medium containing 5% FCS were measured and subtracted from samples of in vitro assays in order to determine genuine TGFβ1 production. Total TGFβ1 in serum samples was measured by acid activation (2.5 N acetic acid/10 µl urea) for 10 min followed by neutralization (2.7 N NaOH/1 µl HEPES). Samples were then diluted 10-fold in PBS/0.05% Tween 20 and measured as described above. The TGFβ1 assay used in this study has no cross-reactivity with TGFβ2, TGFβ3 or other cytokines (BD Biosciences).

DNA extraction and TGFβ1 genotyping

Genomic deoxyribonucleic acid (DNA) was extracted from peripheral blood using the Puregene DNA purification kit (Gentra Systems, Minneapolis, MN) according to the manufacturer’s instructions. TGFβ1 genotyping was by amplification refractory mutation site polymerase chain reaction (ARMS-PCR) analysis for codon 10 and 25 as previously described [8]. Products were separated and visualized by ethidium bromide-stained agarose gel electrophoresis. To avoid incorrect assessment of genotype, all tests were performed in duplicate independently. Negative and positive controls were included in each run to ensure specificity.

Statistical analysis

Data are shown as mean ± s.d. Serum TGFβ1 levels were analysed with the non-parametric Mann–Whitney U-test. Cytokine levels were compared between stimulated and control cultures using the Student’s t-test. P values less than 0.05 were considered significant. The distribution of codon 10 and 25 TGFβ1 polymorphisms was analysed using Fisher’s exact test to identify significant departures from the Hardy–Weinberg equilibrium. Finally, based on the power calculation for the size of our study population, the odds ratio of the difference in genotype frequency had to be less than 0.2–0.25 in order to detect a significant difference (P < 0.05).

Results

Serum levels of TGFβ1

We found that serum levels of TGFβ1 in AS patients were significantly higher than in controls (12.08 ± 3.56 ng/ml vs 4.95 ± 1.20 ng/ml, respectively; mean ± s.d.; P < 0.001). Median serum concentrations of TGFβ1 in our AS patients did not differ significantly between codon 10 and codon 25 genotypes (data not shown).

In vitro stimulation assays

Incubation of AS and normal PBMCs with PHA led to upregulation of TGFβ1, IL-10, TNFα and IFNγ production as assayed by ELISA. When cultured in medium alone, PBMCs from AS patients produced more TGFβ1 than those from normal donors, although this was not significant (P > 0.05). Furthermore, PHA-induced TGFβ1 production was significantly increased in AS patients compared with normal controls (1066 ± 250 pg/ml vs 238 ± 134 pg/ml, respectively; mean ± s.d.; P < 0.05, Fig. 1A). AS PBMCs also secreted more IL-10 upon activation by PHA. In contrast, PHA induced only low and variable levels of TNFα and IFNγ synthesis in AS PBMCs compared with normal controls (Figs 1B–D). These results suggest that AS PBMCs exhibit an up-regulated response to TGFβ1 production but a down-regulated response to TNFα and IFNγ production.

Fig. 1. Production of cytokines by PBMC following stimulation with PHA in both AS and normal controls. PBMCs were purified from healthy individuals (n = 5) or AS patients (n = 6) and cultured for 72 h at 37°C with PHA (10 µg/ml). Cytokine concentrations in the culture supernatant were determined by ELISA. Experiments from each donor were performed in triplicate and graphs show mean ± s.d. from all donors. *P < 0.05, Student’s t-test.
Our study populations with various genotypes are correlated with the platelet TGFβ1 levels. Interestingly, our study showed that PBMCs from AS patients produced significantly higher levels of TGFβ1 following PHA stimulation than healthy controls, suggesting that these cells exhibit an up-regulated response in TGFβ1 production. We also observed an increase in PHA-induced IL-10 production in AS patients. However, only low and variable levels of TNFα and IFNγ synthesis in AS PBMCs were detected compared with normal controls (Fig. 1). Similar findings were observed by Rudwaleit et al. [11], whose study demonstrated lower T-cell production of TNFα and IFNγ in this disease, suggesting that there may be a systemic dysregulation of the cell-mediated immune response in AS.

In contrast to AS, lymphocyte production of total and active forms of TGFβ1 has been shown to be decreased in patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [12]. As the mechanisms involved in this cytokine imbalance in AS are not clear, we compared the frequency of codon 10 and 25 TGFβ1 polymorphisms between AS patients and healthy controls but found no difference in genotype distribution between the two groups. We chose to study the codon 25 TGFβ1 polymorphisms because of their association with diseases such as systemic sclerosis, lung and hepatic fibrosis [13] and codon 10 TGFβ1 polymorphisms because of their association with osteoporosis, spinal osteoarthritis and RA [14]. The TGFβ1 genotype distribution for AS patients in our study was similar to that for the controls (Table 1). Thus, although our AS patients had significantly higher serum levels of TGFβ1 than healthy controls, no correlation was found with codon 10 and 25 polymorphisms of the gene, possibly as a result of ethnic differences. However, given the limited sample size and statistical power in our current study, a much larger cohort is required to confirm our findings.

It is of interest that a recent study in the Finnish population with 437 individuals reported only a weak association between the rare TGFβ1 +1632 T allele and AS (P = 0.04), thus the authors’ conclusion that TGFβ1 polymorphism plays at most a minor role in the pathogenesis of AS and that other genes encoded on chromosome 19 are involved in disease susceptibility [15].

In summary, our data show that AS patients have significantly elevated serum levels of TGFβ1, independent of codon 10 and 25 TGFβ1 gene polymorphisms, and a reduced ability to secrete TNFα and IFNγ in vitro. The mechanisms behind such cytokine imbalance are currently unclear and merit further investigation.

### Acknowledgements
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The authors have declared no conflicts of interest.

### References

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<td>96.9 (93)</td>
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<td>3.1 (3)</td>
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<tr>
<td>CC</td>
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Allele frequency:

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<td>OR (95% CI)</td>
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TABLE 1. TGFβ1 gene polymorphism and susceptibility to AS

CODON 10 TT | 41.5 (30) | 32.2 (31) | 0.346
TC          | 47.1 (34) | 58.6 (56) |         |
CC          | 11.4 (8)  | 9.2 (9)   |         |

Allele frequency:

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<th>Controls (n=96)</th>
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<td>T</td>
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<td>61.5</td>
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<td>C</td>
<td>34.7</td>
<td>38.5</td>
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<td>P (TT + TC vs CC)</td>
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<tr>
<td>OR (95% CI)</td>
<td>0.83 (0.27, 2.61)</td>
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<tr>
<td>P (TC vs CC, TT vs CC)</td>
<td>0.4723, 0.8770</td>
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<td>OR (95% CI)</td>
<td>0.68 (0.21, 2.26), 1.09 (0.33, 3.72)</td>
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NA, not available; OR, odds ratio; CI, confidence interval.

TGFB1 gene polymorphisms and susceptibility to AS

TGFB1 genotyping data are presented in Table 1. No significant difference in codon 25 genotypes was found between AS patients and controls, as both groups predominantly had the GG genotype for codon 25 (95.8 and 96.9% respectively, P = 0.516). No significant difference was found also for codon 10 polymorphisms between patients and controls. Finally, we failed to observe any significant association between TGFB1 codons 10, 25 and disease susceptibility in our local population (data not shown).

Discussion

This study demonstrates that our local southern Chinese AS patients have significantly elevated levels of TGFβ1 in their serum, and also that AS PBMCs have an up-regulated response in TGFβ1 production. Our results do not support the hypothesis of an association between a polymorphism in the signal sequence of the TGFB1 gene and susceptibility to AS. The significance of the elevated serum TGFβ1 levels observed in our study is not known and the precise role of TGFβ1 in AS pathogenesis and disease perpetuation is still not clear.

TGFβ1 plays a critical role in the regulation of inflammatory events, extracellular matrix synthesis and bone modelling and may be important in the pathogenesis of AS and other inflammatory diseases [1, 3, 4]. TGFβ1 stimulates several processes that are critical for tissue repair, including the reduction of pro-inflammatory cytokine production from macrophages, promotion of cell growth and stimulation of extracellular matrix deposition [4, 5]. However, Toussirot et al. [9] failed to observe any association of serum TGFβ1 levels and bone mass in their study, suggesting that TGFβ1 may serve rather as an immunomodulator to counteract the autoimmune nature of this disease. As this cytokine directs IgA switching in B cells the raised IgA levels in AS may be a result of elevated serum TGFβ1 levels [3]. In addition, most TGFβ in serum appears to be derived from platelets, which contain two pools of latent TGFβ1. One pool, containing the latent TGFβ binding protein and the mature TGFβ1 dimer, is released into the serum during clotting [10]. It is not clear whether the differences in the circulating concentration of TGFβ1 among our study populations with various genotypes are correlated with