Cytokine 53 (2011) 306–310

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/issn/10434666

TNF-α −308 genotypes are associated with TNF-α and TGF-β$_1$ mRNA expression in blood leucocytes of humans

Simone Helmig *, Nahid Aliahmadi [1], Peter Stephan [1], Juliane Döhrel, Joachim Schneider

Institut und Poliklinik für Arbeits- und Sozialmedizin, Justus-Liebig-Universität, Aulweg 129, D-35392 Giessen, Germany

ARTICLE INFO

Article history:
Received 28 July 2010
Received in revised form 4 November 2010
Accepted 19 November 2010
Available online 18 December 2010

Keywords:
TNF-α gene polymorphism
TNF-α gene expression
TGF-β$_1$ gene expression
Cytokines
Signalling

ABSTRACT

Aim: Tumor necrosis factor α (TNF-α) influences the pathogenesis of lung-fibrosis and carcinogenesis in normal cells. Polymorphisms of this gene are suggested to be associated with susceptibility to lung-diseases. Additionally TNF-α is postulated to play a significant role in regulating. Transforming growth factor (TGF-β$_1$) expression Therefore we investigated if the TNF-α or TGF-β$_1$ gene expression level is different within the −308 TNF-α genotypes.
Methods: Quantitative Real-time PCR of TNF-α and TGF-β$_1$ was performed in 178 Germans. Calculations of expression were made with the $2^{-\Delta\Delta CT}$ method. Detection of the −308 promoter polymorphism of the TNF-α gene was performed by rapid capillary PCR with melting curve analysis.
Results: The relative TNF-α mRNA expression revealed significant differences between the TNF-α −308 homozygote wild-type G/G (0.00079 ± 0.00011; $n = 113$) and the heterozygote genotype G/A (0.0005 ± 0.00008; $n = 52$; $p = 0.030$) as well as between homozygote wild-type G/G and the homozygote mutant A/A (0.00029 ± 0.00009; $n = 5$; $p = 0.004$). The relative TGF-β mRNA expression showed, similar to TNF-α, the highest mRNA expression was seen within the TNF-α −308 homozygote wild-types, while the lowest mRNA expression lay within the homozygote mutant-types.
Conclusion: Our findings suggest that the G-allele of TNF-α −308 is associated with a significantly higher TNF-α mRNA expression compared to the A-allele and that this also reflects in TGF-β expression. Therefore we support the thesis that TGF-β is regulated by TNF-α.

1. Introduction

Tumor necrosis factor α (TNF-α) a multi-functional, pro-inflammatory cytokine is produced primarily by monocytes, macrophages and lymphocytes. It exhibits many inflammatory effects (e.g. activating neutrophils and mononuclear cells, inducing expression of adhesion molecules, cytokines and chemokines) and is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, and apoptosis.

Single-nucleotide polymorphisms (SNPs) in regulatory regions of cytokine genes have been associated with susceptibility to a number of complex disorders, for review see [1–3]. Variation in the TNF-α promoter region has been found to be associated with susceptibility to several lung diseases such as chronic bronchitis [4], fibrosing alveolitis [5], asthma [6], silicosis [7] and coal workers' pneumoconiosis (CWP) [8], as well as non-small cell lung cancer [9]. The TNF-α −308 promoter polymorphisms is described to be associated with the outcome and progression of asbestos induced fibrosis and related malignancies [10].

The SNP located at nucleotides −308 (rs1800629) of the TNF-α promoter region, is a substitution of adenine for guanine [11]. In literature the allelic types of the −308 polymorphism are referred to as −308G and −308A or TNF1 and TNF2 respectively [6,12].

Additionally in many cell types, there is increasing evidence that TNF-α influences Transforming growth factor (TGF-β$_1$) levels [13–16]. TNF-α is postulated to play a significant role in regulating TGF-β$_1$ expression. In lung fibroblasts, e.g. TNF-α is supposed to induce TGF-β$_1$ via AP-1 activation [17,18]. In lung fibroblasts, for example, TNF-α is supposed to induce TGF-β$_1$ via AP-1 activation [17,18]. A significant association of the relative TNF-α/B2M mRNA expression with the relative TGF-β1/B2M mRNA expression has been demonstrated in 118 Germans [19].

Expression of TNF-α is increased in patients with pulmonary fibrosis and anti-TNF-α agents are therapeutically used in many diseases [20]. Transforming growth factor-β$_1$ (TGF-β$_1$) is a multifunctional cytokine that plays a key role in proliferation and differentiation of cells. Increased levels of TGF-β$_1$ expression is a consistent feature of many fibrotic diseases [21]. The role of a master switch has been attributed to TGF-β$_1$ for induction of fibrosis [22].

Since there is clear evidence for an association of the promoter polymorphism −308 with fibrotic or malignant diseases and

* Corresponding author. Tel.: +49 0 641 99 41315; fax: +49 0 641 99 41309.
E-mail address: Simone.Helmig@arbmed.med.uni-giessen.de (S. Helmig).
[1] Some of the results are included in the thesis of N. Aliahmadi and P. Stephan.

1043-4666/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.cyto.2010.11.018

because of an interrelationship between TNF-α and TGF-β_1 expression we investigated whether TNF-α −308 genotypes are associated with different TNF-α and TGF-β_1 mRNA levels in blood leucocytes of humans.

2. Methods

2.1. Subjects

The study population consisted of a total of 178 Germans (mean age 553 years; min.: 20 years, max.: 91 years). The group comprised 119 male (mean age 57 years; min.: 21, max.: 91) and 59 female (mean age 48 years; min.: 20, max.: 82). Volunteers were personnel of the Universitätsklinikum Giessen und Marburg GmbH, students of the Justus-Liebig-Universität Giessen as well as patients recruited at the Policlinic of the Institute for Arbeits- and Sozialmedizin Giessen, Germany.

All subjects included in this study were interviewed using a questionnaire to obtain information on lifestyle (including a lifetime history of tobacco use) and occupational history. According to their reported smoking habit, patients were classified into smokers, former smokers or never smokers. Individual pack-years (PJ) were calculated. Written informed consent was obtained from all patients before inclusion in the study.

The ethics committees of the university hospital, Giessen, Germany, approved the study (AZ.:75/06).

2.2. Real-time PCR and polymorphism detection

Three millilitre of whole blood was collected by venipuncture in tubes containing EDTA (Sarstaedt, Nümbrecht, Gernmany). Genomic DNA was isolated from whole blood using the VersageneTM DNA Purification Kit (Gentra Systems, Minneapolis, USA). Detection of the polymorphisms was performed by rapid capillary PCR, with melting curve analysis, using fluorescence-labelled hybridisation probes in a LightCycler System (Roche Diagnostics, Mannheim, Germany). The PCR primers as well as the fluorescent-labelled detection probes were synthesized by TIB MOLBIOL (Berlin, Germany). Detection of the TNF-α promoter polymorphisms was performed by a duplex assay as described [10].

2.3. RNA extraction and reverse transcription

White blood cells (WBC) were isolated from 10 ml peripheral blood using Ficoll® (GE Healthcare) as described by the manufacture. Total RNA was isolated from WBCs using a commercial RNA isolation reagent (TRI Reagent®, Sigma) according to the manufacturer's instructions. Isolated RNA was resuspended in 10 μl of RNAse-free water. Each sample was treated twice with 2 μl RNAse-free DNAse 1 unit/μl (Qiagen) for 10 min at 37 °C to eliminate remaining DNA. The prepared RNA was reverse-transcribed as described [23].

2.4. Real-time polymerase chain reaction (PCR)

For quantitative comparison of mRNA levels Real-time PCR was performed using SYBR-green fluorescence in a LightCycler® System (Roche Diagnostic GmbH). Amplification specificity was checked using melting curves. Both negative and positive controls were included in each PCR reaction. All assays were carried out three times as independent PCR runs for each cDNA sample. Gene expression was always related to expression of beta-2-microglobulin (B2M) as housekeeping gene, which is known to be a good choice for normalization of leucocytes expression levels [24]. Calculations of expression was performed with the $2^{-\Delta\Delta CT}$ method according to Pfaffl et al. [25]. The primer sequences and PCR conditions were as published [19]. All measurements were made without information about the origin of the samples.

2.5. Statistical analysis

Smokers were considered current smokers at time of diagnosis. Ex-smokers were all people who had ever smoked. Information was collected on the usual number of cigarettes smoked per day, the age at which the subject started smoking and, if the person was an ex-smoker, the age at which the subject stopped smoking. One PJ was defined as smoking 20 cigarettes daily over one year.

Allelic and genotype frequencies were obtained by direct counting. Hardy–Weinberg equilibrium was assessed by a χ^2 test with 1° of freedom.

All statistical analyses were performed using the statistical software package, SSPS 15.0 (SPSS Inc., Chicago, IL, USA). Data within the 95% CI were used for analysis. Results are expressed as mean ± standard error of the mean (SE). Means in different subgroups were analysed by Student's *t*-test and by one-way ANOVAs followed by Duncan- and Student–Neumann–Keul's post hoc analysis and Mann–Whitney *U* test. Peason's correlation coefficients were used to examine the relation between levels of mRNA expression. A value of $p < 0.05$ was regarded as significant.

3. Results

3.1. Genotype and allele frequencies of TNF-α promoter polymorphisms

The frequency of the rare TNF-α(−308) A-allele was 0.18 in the investigated population. The genotype frequency of the homozygote wildtype (G/G) was 0.673, the heterozygote (G/A) was 0.295 and the homozygote mutant (A/A) was 0.032.

The studied population, including controls, did not deviate from Hardy–Weinberg equilibrium ($\chi^2 = 0.15$ for TNF-α(−308) polymorphism).

3.2. TNF-α(–308) promoter polymorphisms and TNF-α mRNA expression

To investigate the functional impact of the TNF-α(−308) allelic variants the quantitative TNF-α mRNA expression among the different genotypes for TNF-α −308 polymorphism (G/G, G/A; A/A) was analysed. The determined relative TNF-α expression ranged from 0.00001 to 0.05575 with a median at 0.00034. TNF-α mRNA expression did not show any significant association with the smoking habits ($p = 0.513$), age ($p = 0.353$), gender ($p = 0.703$) or origin of sample ($p = 0.813$).

Comparing the means ± standard error of the relative TNF-α mRNA expression revealed significant differences between the homozygote wild-type G/G (0.00079 ± 0.00011; $n = 113$) and the heterozygote genotype G/A (0.0005 ± 0.00008; $n = 52$; $p = 0.030$) as well as between homozygote wild-type G/G and the homozygote mutant A/A (0.00029 ± 0.00009; $n = 5$; $p = 0.004$). This suggests that the G-allele is associated with a significantly higher TNF-α mRNA expression compared to the A-allele Fig. 1).

3.3. TNF-α(–308) promoter polymorphisms and TGF-β_1 mRNA expression

Since there is increasing evidence that TNF-α influences TGF-β_1 expression, we checked if the different TNF-α mRNA expression of the three TNF-α −308 genotypes are reflected in the TGF-β expression as well.