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Clinical Importance of Transforming Growth Factor-β but Not of Tumor Necrosis Factor-α Gene Polymorphisms in Patients with the Myelodysplastic Syndrome Belonging to the Refractory Anemia Subtype

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Key Words

Gene polymorphisms · Myelodysplastic syndrome · Refractory anemia · Transforming growth factor-β · Tumor necrosis factor-α

Abstract

Objectives: Tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) are cytokines that play key roles in the myelodysplastic syndrome (MDS). There have been several reports on the presence of genetic polymorphisms in the DNA sequence encoding the leader sequence of the TGF-β1 protein, located in codon 10 in exon 1 and in the –308 promoter region of TNF-α. The objective of this study was to investigate the association between TNF-α and TGF-β1 gene polymorphisms and the susceptibility to MDS and the progression of the disease among patients with MDS belonging to the refractory anemia (RA) subtype. ***Methods:*** The diagnosis of MDS (n = 50) was based on the FAB criteria. The TNF-α genotypes were analyzed by PCR-RFLP and the TGF-β genotypes were analyzed using an amplification refractory mutation system. ***Results and Conclusions:*** Compared with healthy control subjects, patients with RA showed no significant deviations in genotype or allele frequencies of TNF-α. The TT homozygosity at codon 10 of TGF-β1 was significantly higher among patients with bi- or pancytopenia (severe group) than in the patients with anemia only (mild group; odds ratio = 6.99, p = 0.003). These findings suggest that the TGF-β1 gene polymorphism in codon 10 and the –308 TNF-α gene polymorphism do not predispose to the development of RA, but the TGF-β1 gene polymorphism may affect disease progression.

Introduction

The myelodysplastic syndrome (MDS) comprises a distinct, albeit heterogeneous group of hematopoietic disorders characterized by ineffective hematopoiesis and an increased propensity to marrow failure and leukemic transformation. The natural history is highly variable. The current classification systems of MDS (FAB and

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WHO) are based on the morphological features of the bone marrow and the blood [1, 2]. Cytogenetic and molecular studies support the concept that the stepwise accumulation of genomic damage in hematopoiesis is central in the natural disease course of MDS, but it is generally accepted that the hematopoietic microenvironment is also active in the hematopoietic failure. Abnormal cytokine production, autoreactive T lymphocytes and an altered interaction between the progenitor cells and the extracellular matrix can all promote apoptosis, which has a central role in impaired hematopoiesis in MDS. A number of investigators have demonstrated an excessive production of proapoptotic cytokines in the bone marrow of MDS patients. Several studies have stressed the importance of increased levels of tumor necrosis factor-α (TNF-α) in the serum and bone marrow in promoting apoptosis [3, 4]. Transforming growth factor-β (TGF-β) is another cytokine which is generally considered to be a negative key regulator of hematopoietic stem and progenitor cells [5–7]. Moreover, one of the main new directions in the treatment of MDS is the use of compounds which exert inhibitory activity on proapoptotic cytokines [8–10].

The regulatory and coding regions of cytokine genes are relatively polymorphic and may therefore be useful in disease association studies. It has been demonstrated that the production of TNF-α or TGF-β1 varies from individual to individual and partly depends on the polymorphisms of these genes. The most widely investigated polymorphism of the TNF-α gene is the G→A transition in the –308 promoter region [11]. It has been shown that an A nucleotide at position –308 is associated with increased transcription and production of TNF-α [12, 13]. Further, several polymorphisms have been described in the TGF-β1 gene, including a T→C transition at nucleotide 29, in the region encoding the signal sequence, which results in a leucine-proline substitution at the 10th amino acid [14]. It has been shown that TT homozygous genotypes are high TGF-β1 producers [15–17]. The correlation between the TNF-α gene polymorphism [18–22] or the TGF-β1 gene polymorphism [23–26] and the disease status has been studied in a diverse range of diseases, the results suggesting that the gene polymorphisms of TNF-α or TGF-β may provide a rational indicator of susceptibility to or prognosis of the disease. Since most of our MDS patients belonged to the refractory anemia (RA) subtype, in the present study, we have investigated whether a TNF-α gene polymorphism in the –308 promoter region or a TGF-β1 gene polymorphism in codon 10 are associated with the development and severity of MDS in patients belonging to the RA subtype.

Patients and Methods

Patients

Between May 2001 and January 2004, a total of 50 cases with RA documented by marrow biopsy were enrolled (12 men and 38 women, age 72.48 ± 1.02 years, range 55–87 years). The diagnosis of MDS was based on the FAB criteria [27]. In 4 of the patients, hypoplastic MDS was documented; in all of the other patients, the bone marrow was normocellular or hypercellular, satisfying the morphologic features of RA according to the FAB and WHO criteria (<5% blasts and <15% ringed sideroblasts). Neither a fibrotic form nor secondary MDS was diagnosed. Cytogenetic analysis revealed a normal karyotype in 38 cases, no mitosis in 5 cases, 5q- in 1 case and 8 chromosome abnormalities in 6 cases. The median follow-up was 34.5 months (range: 14–62 months). None of the patients had received specific therapeutic agents prior to the study. They were supported only by red blood cell transfusion. Patients were divided into two groups: a mild group comprising patients with anemia only (hemoglobin <100 g/l; n = 30), and a severe group consisting of patients with bi- or pancytopenia (neutrophil count <1 G/l, platelet count <100 G/l; n = 20). Leukemic transformation occurred in 3 patients; another 4 patients died due to MDS, without leukemic transformation.

A control group of 74 healthy blood donors (age, 41.31 ± 1.47 years, range 21–73 years; 32 men and 42 women) was investigated. All patients and controls were of adult Hungarian ethnic origin and resided in Hungary.

This study was performed in accordance with the ethical standards laid down in the most recent version of the 1964 Declaration of Helsinki. The ethical committee of the participating university had approved the study. Informed written consent was obtained in advance from each of the patients.

DNA Extraction

Genomic DNA from whole blood containing EDTA was extracted by a standard technique (High Pure PCR Template Preparation Kit, Roche Diagnostics, Mannheim, Germany). The analysis of the polymorphism was based on polymerase chain reaction techniques performed in a thermal cycler (GeneAmp PCR System 2700, Applied Biosystems, Foster City, Calif., USA).

TNF-α Genotype Assay

The G→A transition at position –308 in the promoter region was analyzed by PCR-RFLP [11]. A single base change at the 3′ end of primer A1 (underlined) was required for the formation of an *Nco*I (Fermentas, Vilnius, Lithuania) recognition sequence CCATGG (instead of GCATG originally found on the investigated gene; primer A1: 5′-AGGCAATAGGTTTTGAGGGCCAT-3′ and primer A2: 5′-TCCTCCCTGCTCCGAT TCCG-3′) [11]. The reaction mixture of 100 μl contained 100 ng of genomic DNA, 20 pmol of the A1 and the A2 primer, respectively, 2.5 U Taq DNA polymerase, 1.5 m*M* $MgCl_2$, 1× PCR Taq polymerase buffer + $(NH_4)_2SO_4$ (Fermentas) and 25 m*M* of each dNTP (Fermentas). The PCR conditions were as follows: initial denaturation at 94°C for 3 min; 36 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 5 min. The amplified product was digested with the endonuclease *Nco*I and analyzed on a 12% polyacrylamide gel. The TNF G allele gives two fragments of 87 and 20 bp, while the TNF A allele gives a single, 107-bp fragment.

TGF-β Genotype Assay

The defined single-nucleotide polymorphism T^{29}-C in exon 1 of the human TGF-β1 gene was determined with an amplification refractory mutation system with a generic primer (sense), (5′-TCC-GTGGGATACTGAGACACC-3′), and with two allele-specific antisense primers, differing from each other in only one base at the 3′ end: primer C: 5′-GCAGCGGTAGCAGCAGCG-3′ and primer T: 5′-AGCAGCGGTAGCAGCAGCA-3′ [28]. The reaction mixture of 50 μl contained 100 ng of genomic DNA, 20 pmol of each the sense and the antisense primer, 1.25 U Taq DNA polymerase, 1.5 m*M* $MgCl_2$, 1 × PCR Taq polymerase buffer with $(NH_4)_2SO_4$, and 25 m*M* of each dNTP (Fermentas). The thermocycling procedure was as follows: initial denaturation at 94°C for 5 min; 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were analyzed by 1.5% agarose (Sigma-Aldrich, St. Louis, Mo., USA) gel electrophoresis. The expected size of the specific amplification product was 241 bp. Samples from 2 known homozygotic individuals and 1 heterozygotic individual, confirmed by sequencing, were included in each reaction. Sequencing was performed with an automated sequencer (ABI Prism; Applied Biosystems, Foster City, Calif., USA).

Statistical Analysis

Statistical analyses for comparisons of allele and genotype frequencies between groups were made using the χ^2 test, and Fisher's exact test if one cell had $n < 5$. A significance level of $p < 0.05$ was considered statistically significant. For comparison of age and sex between the patients and the controls, the Mann-Whitney U test and Fisher's exact test were used. Statistical calculations were performed with the GraphPad Prism4 statistical program.

The genotype frequencies for each polymorphism were tested for deviation from the Hardy-Weinberg equilibrium by means of the χ^2 test, using one degree of freedom.

Results

The distributions of genotypes and alleles for the different polymorphisms were consistent with those predicted by the Hardy-Weinberg equilibrium in the patients and in the controls. The two groups were matching according to sex (Fisher's exact test: $p = 0.058$), but there was a significant difference in the mean age between the controls and the patient group (Mann-Whitney U test: $p < 0.01$).

The –308 Promoter Polymorphism of TNF-α

The genotypic distribution of the TNF-α gene –308 promoter G→A polymorphism is shown in table 1. There were no significant differences in the TNF-α –308 promoter genotypic distribution between the patients with RA (GG, GA and AA = 62.0, 32.0 and 6.0%, respectively) and the healthy controls (GG, GA and AA = 68.9, 28.3 and 2.8%, respectively). Similarly, there were no differences in the distribution of TNF-α –308 G→A genotypes between the RA patients with anemia only (mild group; GG, GA and AA = 63.3, 33.3 and 3.3%, respectively) and those with bi- or pancytopenia (severe group; GG, GA and AA = 60.0, 30.0 and 10.0%, respectively)

Table 1. Distribution of TNFα (–G308A) genotypes in the MDS patients belonging to the RA subtype and in the healthy controls

	TNF-α (–G308A) genotypes			p
	GG n (%)	GA n (%)	AA n (%)	
Patients with RA (n = 50)	31 (62.0)	16 (32.0)	3 (6.0)	
Mild (n = 30)	19 (63.3)	10 (33.3)	1 (3.3)	0.650[a]
Severe (n = 20)	12 (60.0)	6 (30.0)	2 (10.0)	
Controls (n = 74)	51 (68.9)	21 (28.3)	2 (2.8)	

The mild group comprised patients with anemia only and the severe group patients with bi- or pancytopenia.

[a] $\chi^2 = 2.466$, d.f. = 4; comparisons between the mild group, the severe group and the controls.

Table 2. Distribution of TGF-β T^{29}-C (Leu10→Pro) genotypes between the MDS patients belonging to the RA subtype and the healthy controls

	TGF-β (T^{29}-C) genotypes			p
	TT n (%)	CT n (%)	CC n (%)	
Patients with RA (n = 50)	24 (48.0)	19 (38.0)	7 (14.0)	0.031[a]
Mild (n = 30)	9 (30.0)	16 (53.3)	5 (16.7)	0.007[b]
Severe (n = 20)	15 (75.0)	3 (15.0)	2 (10.0)	0.003[c]
Controls (n = 74)	33 (44.6)	28 (37.8)	13 (17.6)	

[a] $\chi^2 = 10.58$, d.f. = 4; comparisons between the mild group (patients with anemia only), the severe group (patients with bi- or pancytopenia) and the controls.

[b] $\chi^2 = 9.923$, d.f. = 2; statistical analysis was performed between the TT and CT + CC genotypes; comparisons between the mild group, the severe group and the controls.

[c] Fischer's exact test: odds ratio = 6.99; statistical analysis was performed between the TT and CT + CC genotypes; comparison between the mild group and the severe group.

The T^{29}-C Polymorphism in Codon 10 of TGF-β1

The genotypic distribution of the T^{29}-C polymorphism of the TGF-β1 gene is shown in table 2. No significant difference in genotypic distribution was found

between the RA patients overall and the healthy controls. A significant difference was observed, however, when the genotypes were taken into consideration within the two subgroups of RA ($\chi^2 = 10.58$, $p = 0.031$). To elucidate the reason for this significance, we compared the number of TT homozygotes (high TGF-β-producing phenotype) and C carriers (CT and CC) among the patients with mild and severe cytopenia forms of RA and the healthy controls. A significant difference was observed when the TT homozygotes and C carrier genotypes were compared within the two subgroups of RA together with the healthy controls ($\chi^2 = 9.923$, $p = 0.007$). There was also a considerable difference in the frequency of the TT genotype between the two RA subgroups. This was due to the overrepresentation of the TT genotype in the patients with bi- or pancytopenia [severe group; TT = 75.0% (severe) vs. TT = 30.0% (mild), $p = 0.003$, odds ratio = 6.99, 95% confidence interval = 1.949–25.144]. Alternatively, the C carrier status (representing the CT and CC genotypes) was more frequent among the RA patients with anemia only (mild group). Since we had only 4 patients with the hypoplastic form of MDS, it was not meaningful to perform any statistical analysis concerning marrow cellularity. Nevertheless, the genotypic analysis revealed that 2 of these patients had the TT homozygote genotype.

Discussion

During the past few years, major progress has been made toward an understanding of the pathological processes of MDS. Genetic abnormalities or altered gene expressions leading to the suppression of hematopoiesis may also be responsible for the development of cytopenia. One of the genetic factors may be an aberration of cytokine regulation and cytokine production, which can lead to an increased apoptosis of hematopoietic progenitor cells, and aberrant hematopoiesis. The excessive production of growth-inhibitory cytokines such as TNF-α in patients with MDS has been described [3, 4, 29]. Recent data indicate that TGF-β inhibits myeloid, erythroid, megakaryocytic and multilineage colony formation [5–7]. Accordingly, it appeared plausible to hypothesize that polymorphisms of the TNF-α [30] or TGF-β1 genes may be correlated with MDS or the severity of the disease.

In our study, however, no significant difference in the –308 TNF-α polymorphism was observed regarding the G or A alleles when the forms of RA were compared with each other or with the controls.

TNF-α also has an apoptotic effect toward hematopoietic cells. However, the distribution of the –308 TNF gene polymorphism was similar among the patients in the two RA subgroups. Comparison of the genotype frequencies in the patients with RA suggests that the –308 G/A polymorphism of TNF-α does not affect susceptibility to the disease. Within the limits of the currently available evidence, we presume that this polymorphism determining TNF-α secretion might be minimized through the secretion of other factors in MDS. There are several potential reasons for failing to observe any association between the –308 TNF-α polymorphism and RA. The –308 TNF-α polymorphism would appear to occur at a transcription factor binding site in the TNF-α promoter and hence affects protein binding, thereby altering transcriptional activity. The transcriptional and translational regulation and prosttranslational protein processing are major steps involved in protein expression. TNF protein expression is probably not exclusively regulated at the transcriptional level. In complex biological systems, the effect of a single gene polymorphism determining cytokine secretion may be minimized through the interaction of other factors.

TGF-β isoforms are translated as prepropeptide precursors with an N-terminal signal peptide followed by the prodomain and mature domain, and are secreted as biologically latent forms. The Leu10→Pro-(T^{29}-C) polymorphism in exon 1 of the TGF-β1 gene is located in the signal peptide sequence that is cleaved from the TGF-β1 precursor at the level of codon 10 [31]. This polymorphism at codon 10 (within the hydrophobic α-helix of the signal sequence) involves the replacement of a hydrophobic leucine residue with a small, neutral proline residue. Such a change would alter the overall hydrophobicity of the core transport sequence and disrupt the α-helical structure of the region, thereby altering its ability to direct protein transport across the endoplasmatic reticulum [14]. The T^{29}-C polymorphism of the TGF-β1 gene does not result in a difference in the mature TGF-β1 protein; merely the TGF-β1 protein level is reduced both in vitro and in vivo [15–17].

On the other hand, Yokota et al. [32] reported that the T allele of T^{29}-C was linked to a lower production of TGF-β1. The influence of the allele at codon 10 on TGF-β production is possibly more variable, due to the cross-modulating interfaces between the signaling networks within the cells. In this study, we have shown that in patients with RA the TGF-β 'high-producer genotype' TT causes a 6.99-fold increased risk of bi- or pancytopenia compared with the C-carriers. Patients carrying the C 'protec-

tive' allele, with a concomitantly lower TGF-β1 production, have only anemia. As TGF-β1 is a member of the proapoptotic cytokines, it is very likely that patients with the low-producing genotypes (CC and CT) may have a better chance of a less severe cytopenia in MDS. Although we could not find genotypic differences between the RA patients overall and the healthy controls, we observed that the genotype of the TGF-β1 T^{29}-C gene differed significantly between the severe group and the mild group. Although the mean age of the study groups was significantly different, it is unlikely that this age difference would introduce bias into the study because significant differences in the distribution of TGF-β genotypes were only found among the RA subgroups. These findings indicate that the polymorphism of the TGF-β T^{29}-C gene itself is not directly involved in the susceptibility to RA, but rather affects the outcome of the disease.

Overall, the polymorphism in the TNF-α gene in the –308 promoter region does not have a principal role in the regulation of the outcome of RA patients. In contrast, our findings of an association between the severe form of RA and TT homozygosity suggest that future studies should focus on the role of TGF-β in RA, and further independent studies with more patients are needed to confirm our observations. Following these pilot experiments, further investigations on the polymorphisms of the TGF-β1 genes should be included in these investigations.

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