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Association of interferon-γ +874A polymorphism with reduced long-term inflammatory response in haemodialysis patients

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Abstract

Background. We have studied the effects of interferon (IFN)-γ allelic variations on expression levels of pro- and anti-inflammatory cytokines and on long-term inflammatory status in haemodialysis patients.

Methods. Genotyping was performed in 123 patients for single nucleotide polymorphisms in the first intron of the IFN-γ gene (+874 T/A). They were prospectively followed for 2 years. Cytokine mRNA levels in whole blood cells (detected by real time (RT)-PCR technique) and serum C-reactive protein (CRP) concentrations were compared in patient groups with different IFN-γ genotypes. Serum CRP was evaluated every month and inflammatory state was defined as percent of abnormal values (above 5 mg/l) over total determinations. Of the total, 102 patients survived and completed 24 ± 1 monthly CRP determinations. The IFN-γ ±874 A/A, 'low-producer' genotype was associated with decreased ($P < 0.05$) mRNA levels of IFN-γ and of interleukin-6 and with a lower ($P < 0.05$) frequency of CRP elevation (37 ± 6%) than the ±874 A/T and T/T, 'intermediate and high-producer' genotypes (59 ± 6%, and 60 ± 5%, respectively). The mRNA levels of tumor necrosis factor-α, IL-10 and of transforming growth factor-β1 were not different in the three groups of patients. Pooled analysis in deceased (10 ± 3 monthly CRP determinations) and survived patients confirmed the results obtained in the patients who completed the follow-up period.

Conclusions. The 'low-producer' IFN-γ +874 A/A genotype was associated with a preventive effect on long-term CRP elevation in haemodialysis patients possibly mediated by decreased gene expression of IFN-γ and IL-6.

Keywords: chronic renal failure; C-reactive protein; haemodialysis; inflammation; interferon-gamma; polymorphisms

Introduction

Uraemic patients on haemodialysis often show signs of chronic inflammation even in the absence of acute complications [1,2]. The causes of inflammation in dialysis have been studied in detail [1,2]. Uraemia and blood-membrane contacts are the most relevant factors that induce changes in cytokine secretion. Nonetheless, the degree of systemic inflammatory response activation exhibits great inter- and intra-individual variability that cannot be explained only by renal disease, dialysis or other clinical inflammatory events [3].

The inflammatory response is modulated by the balance between proinflammatory and anti-inflammatory mediators [2]. Interferon (IFN)-γ is a pro-inflammatory cytokine playing a pivotal role in host defence. This cytokine has the potential to direct the inflammatory response by upregulating a variety of pro-inflammatory mediators including TNF-α and IL-6 [4]. Moreover, data suggest that IFN-γ may also be able to directly enhance activation of the pro-inflammatory nuclear transcription factor-κB (NF-κB) under certain conditions [5]. According to these functional characteristics, IFN-γ bioactivity has been identified as a key mediator in several models of inflammatory diseases [4].

After stimulation, cytokine production varies widely among individuals. Polymorphic bases in the promoter or coding regions of cytokine genes lead to high or low

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productions of these mediators. Individuals with certain genotypes are therefore commonly referred to as low, intermediate, or high cytokine producers, depending on their *in vitro* production rate [6]. Studies in healthy subjects and in patients with different chronic inflammatory diseases have recently shown that selected polymorphisms of the IL-6, TNF-α and IL-10 genes are functionally relevant to determine not only an increased cytokine expression but also inflammatory process activation [7–10]. In patients with end-stage renal disease on haemodialysis, single nucleotide polymorphisms in the promoter region of the pro-inflammatory cytokines IL-6 and TNF-α have been found to be strictly associated with clinical and biochemical indices of comorbidity [10]. The relationships between genotypes of the anti-inflammatory cytokine IL-10 and the acute phase response have been studied in patients with chronic renal failure on haemodialysis [7]. The IL-10 'low-producer' genotype was more permissive for frequent elevations of C-reactive protein (CRP) and was significantly associated with a greater frequency of cardiovascular events [11]. In addition, the combinations of the IL-6 and TNF-α high-producer and of the IL-10 low-producer genotypes were associated with the poorest indices of comorbidity and functional status in haemodialysis patients [10].

Despite the key role of IFN-γ in the inflammatory response regulation, data on the impact of its genetic variation on inflammatory markers are scarce. IFN-γ is encoded by a single gene mapped on chromosome 12 (12q15) [12]. In the first intron of the IFN-γ gene, there is a CA repeat polymorphism that affects transcription. Moreover, an adenine (A) to timine (T) transition at position +874 (intron 1) has been associated with increased IFN-γ expression [12]. The transcription factor NF-κB binds preferentially to the +874T allele [5]. This preferential binding suggests that genetically determined variability in IFN-γ expression might be important for the inflammatory response activated through the NF-κB pathway. The aim of this study was to assess the effects of IFN-γ allelic variation on the overall 2-year inflammatory status in haemodialysis patients as well as on expression levels of IFN-γ and of other pro- and anti-inflammatory cytokines.

Subjects and methods

A cohort of 127 subjects (69 males and 58 females) with chronic renal failure was randomly sampled from the haemodialysis patients in the Nephrology and Dialysis Unit of Cattinara Hospital of Trieste, Italy. Patients gave informed consent. The study was conducted according to the Declaration of Helsinki. Haemodialysis modalities or type of renal disease were not considered as exclusion criteria. Patients with ongoing immunosuppressive treatment or malignancy were excluded. These patients were on haemodialysis since 78 ± 8 (mean ± SEM) months, their age was 67 ± 1 years. All patients were on haemodialysis treatment with a three-times-per-week schedule. Twenty-one patients had type 2 diabetes mellitus. Primary renal diseases of patients were as follows: hypertensive and ischaemic nephropathy $n = 38$; chronic glomerulonephritis $n = 30$; tubulointerstitial nephropathy $n = 17$; diabetic nephropathy $n = 10$; polycystic kidney disease $n = 8$; in 34 patients the etiology of renal disease was unknown. Base-line examinations in 1999–2000 were followed by monthly examinations for 2 years, in which CRP concentration was determined in serum. Patients were not excluded from the analysis if complication developed after the initiation of dialysis. Twenty-five patients died during the observation period and completed only 10 ± 3 monthly examinations. Of the total, 102 patients survived and completed 24 ± 1 monthly CRP determinations. In the patients who completed the observation period, a blood sample was taken in the post-absorptive state to determine cytokine mRNA levels in circulating blood cells. IFN-γ genotype frequencies were determined in the 102 patients who survived, in 21 deceased patients and in 54 healthy, unrelated subjects, mainly composed of blood donors matched for the same ethnic origin of patients. IFN-γ genotype frequencies have not been determined in four deceased patients.

Cytokine genotyping

Cytokine genotyping assay for IFN-γ was based on the PCR-SSP methodology [13]; briefly, it provides sequence-specific oligonucleotide primers for amplification of selected IFN-γ alleles by the PCR reaction. The control primer pair amplifies a conserved region of the humanβ1-globin gene, which is present in all DNA samples and is used to verify the integrity of the PCR reaction. DNA was isolated from EDTA-collected peripheral whole blood using standard laboratory techniques. The analysis of cytokine genotype was performed using the Cytokine Genotyping Primer Pack of the One Lambda, Inc. (CA). 100 ng of DNA were amplified following the PCR reaction profile described in the cytokine genotyping kit protocol. After the PCR process, the amplified DNA fragments were separated by a 2.5% agarose gel and visualized by staining with ethidium bromide. The results were interpreted using a worksheet provided with the product.

Cytokine mRNA quantification

Total RNA was extracted from either 3 ml of whole blood taken in the post-absorptive state at the end of the 2-year follow-up period as previously described [14]. The quality and integrity of total RNA was evaluated by both gel electrophoresis and spectrophotometric determination. All samples were treated by RNAse and DNAse I (10 U/l) (Boehringer Mannheim, Mannheim, Germany) and subsequently extracted with phenol/chloroform/isoamylic alcohol [14]. For each sample, 3 μg of total RNA were incubated together with 100 ng of the oligo dT NOT18 (Pharmacia, Uppsala, Sweden) for 10 min at 68°C and then reverse transcribed in a final volume of 50 μl at 42°C for 1 h using 10 U of MMLV reverse transcriptase (Gibco BRL Life Technologies LTD, Paisley, Scotland) in the presence of an RNAse inhibitor (Gibco BRL). cDNA were then subjected to quantitative RT-PCR (Applied Biosystems-Applera Corporation, USA). TNF-α cDNA amplifications were performed using 3 μl of the RT reaction with the following primers: sense primer 5′-GCAGGTCTACTTTGGGATCA TTG-3′ (nucleotides 757–779, M10988 file in GenBank)

and antisense primer 5′-GCGTTTGGGAAGGTTGGA-3′ (nucleotides 800–817, M10988 file in GenBank); the following specific probe 5′-CTGTGAGGAGGACGAAC-3′ (nucleotides 782–799) with 5′ FAM label and 3′ TAMRA quencher was used. IL-6, IFN-γ, IL-10, TGF-β1 and human glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA amplifications were performed using kits (Applied Biosystems-Applera Corporation, USA) containing mixed primers and probes for human IL-10, human IFN-γ, human IL-6, human TGF-β1 and human GAPDH. A 20× Assays-on-Demand Gene Expression Assay Mix (Applied Biosystems-Applera Corporation, USA) was used for all the amplification reactions according to manufacturers' instructions. Quantitative RT-PCR was performed using the TaqMan chemistry and the 7900/HT sequence detection system (Applied Biosystems) as it follows. Samples were submitted to 40 cycles of amplification with enzyme activation at 50°C for 2 min, pre-denaturation at 95°C for 10 min, denaturation at 95°C for 0.15 s, annealing and extension at 60°C for 60 s. The quantities of the amplified products were based on the fluorescent levels of each sample which were subsequently converted into number of molecules according to a standard curve. Cytokine mRNA amounts are expressed as fraction of GAPDH used as a reference gene [15].

C-reactive protein

CRP was detected by conventional immunoturbidimetric method (CRPLX Tina-quant® Roche, Diagnostics) using automated analyser systems every month in all patients. The overall level of systemic inflammation was expressed as percent of altered CRP values over the total number of determinations. An abnormal serum CRP concentration was defined as higher than 5 mg/l according to the laboratory reference values at the University of Trieste, Cattinara Hospital.

Statistics

Data are presented as mean ± SEM. Results in different IFN-γ genotype groups were compared by one-way analysis of variance or *t*-test where appropriate. Cytokine mRNA levels in different IFN-γ genotype groups were compared after logarithmic transformation. Results were regarded as significant at $P < 0.05$. The primary goal of this study was to determine the influence of IFN-γ genotype on long-term systemic inflammation, expressed as percent of altered CRP values over the total number of monthly determinations, during a 2-year follow-up period. Thus, statistical analysis was performed first in the patients who survived the observation period and completed the same number of monthly CRP determinations. Then, the results were confirmed by including in the analysis the patients who died and completed a lower number of monthly CRP determinations.

Results

The allele frequencies of the IFN-γ gene polymorphism at position ±874 in the 102 dialysis patients who completed the 2-year follow-up period are shown in Table 1. The allele frequencies in 21 of the 25 patients who died before the end of the follow-up period were not significantly different to those of the surviving patients, i.e. A/A, $n = 5$; A/T, $n = 11$; T/T, $n = 6$. Results in IFN-γ allele frequencies of the patients were similar to those of the control population (data not shown) and were as expected from the Hardy–Weinberg law. Table 1 also shows the characteristics of the groups of patients who completed the 2-year follow-up period with the ±874 A/A 'low-producer', A/T 'intermediate producer' and T/T 'high producer' genotypes. The three groups were matched for age, sex distribution, body mass index, haemodialysis duration and prevalence of type 2 diabetes mellitus.

Table 1. Patient[a] characteristics according to IFN-γ gene polymorphism at position +874

	A/A	A/T	T/T
n	28	52	22
Gender (% male)	55	55	52
Age (years)	65 ± 2	67 ± 2	69 ± 2
BMI (kg/m^2)	25 ± 1	25 ± 1	25 ± 1
Months on haemodialysis	70 ± 14	93 ± 18	59 ± 11
Diabetic patients (%)	18	22	20

[a]Data of patients who completed the 2-year follow-up period. Allele frequencies in 21 deceased patients were as follows: A/A: 5; A/T: 11; T/T: 6. BMI: Body Mass Index.

Table 2. Influence of IFN-γ gene polymorphism at position +874 on cytokine mRNA levels in blood cells of haemodialysis patients at the end of the 2-year follow up period

	A/A	A/T	T/T
IFN-γ	1638 ± 337*	22 623 ± 13 508	44 832 ± 27 170
IL-6	674 ± 132**	5082 ± 3113	23 087 ± 13 207
TNF-α	15 ± 4	32 ± 21	8 ± 1
IL-10	19 ± 3	23 ± 3	29 ± 6
TGF-β	2026 ± 480	3243 ± 1203	4483 ± 2717

Cytokine mRNA levels are expressed as fraction of GAPDH mRNA content in white blood cells multiplied by 10^7.
*$P < 0.05$, A/A *vs* A/T and T/T groups.
**$P < 0.05$, A/A *vs* pooled A/T and T/T groups.

Table 2 shows the values of selected pro- and anti-inflammatory cytokines mRNA content in white blood cells of the groups of patients who completed the 2-year follow-up period with the IFN-γ ±874 A/A 'low-producer', A/T 'intermediate producer' and T/T 'high producer' genotypes. As expected, IFN-γ expression was lower in the A/A 'low-producer' genotype than in both the A/T 'intermediate producer' and T/T 'high producer' genotypes. IL-6 mRNA levels were also lower in the A/A 'low-producer' genotype group than in the pooled groups with the A/T 'intermediate producer' and the T/T 'high producer' genotypes. In contrast, mRNA levels of TNF-α, IL-10 and TGF-β1 were not significantly different in the three groups of patients.

CRP concentration was determined 24 ± 1 times in the patients who completed the follow-up period,

whereas it was determined only 10 ± 3 times in the patients who died during the observation period. In all patients, the percentage of altered CRP individual values over the total number of determinations exhibited a great inter-individual variability. In the patients who completed the follow-up period, the average value of this indicator of overall 2-year inflammatory response was 54% (the median was equal to 53%) with a coefficient of variation of 60%. The patients who died before the end of the observation period exhibited 67% of altered CRP values over the total number of determinations. At the end of the follow-up period, absolute CRP value tended ($P < 0.01$) to be lower in the IFN-γ A/A 'low producer' genotype group (9.3 ± 2.3 mg/l) than in the A/T 'intermediate producer' (20.1 ± 5.0 mg/l) and the T/T 'high producer' (18.9 ± 4.7 mg/l) genotype patients. Figure 1 shows the influence of IFN-γ gene polymorphism at position ±874, on the percentage of altered PCR individual values over the total number of monthly determinations in the patients who completed that 2-year follow-up. This indicator of long-term inflammatory response was significantly lower in the IFN-γ A/A 'low producer' genotype group than in the other two groups with the A/T 'intermediate producer' and the T/T 'high producer' genotypes (Figure 1). In addition, we have determined the influence of IFN-γ gene polymorphism on long-term inflammatory response in pooled patients who survived and deceased patients. The analysis in pooled patients confirmed that the percentage of altered CRP values over the total number of monthly determinations was significantly lower in the IFN-γ A/A 'low producer' genotype group (38 ± 6 mg/l) than in the other two groups with the A/T 'intermediate producer' (63 ± 4 mg/l) and the T/T 'high producer' (60 ± 5 mg/l) genotypes.

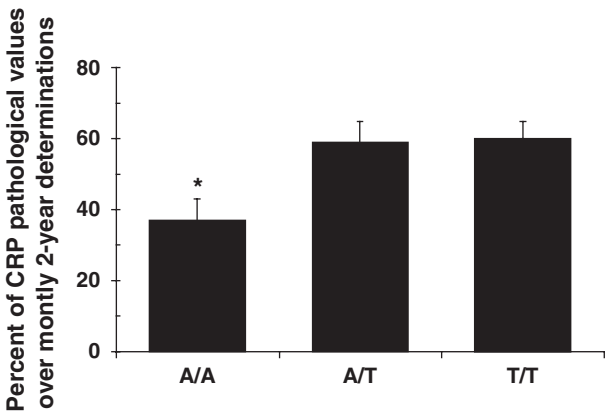

Fig. 1. Influence of IFN-γ gene polymorphism at position +874 on the percentage of altered CRP individual values over the total number of monthly determinations in 2 years. $*P < 0.05$, A/A *vs* A/T and T/T groups.

Discussion

Haemodialysis is associated with a low-grade systemic inflammation that reflects the unbalanced expression of pro- and anti-inflammatory cytokines and contributes to the progression of atherosclerotic vascular disease and malnutrition. CRP is one of the best indicators of the response to inflammation. Elevated CRP levels have been described in a significant proportion of chronically uraemic patients undergoing haemodialysis and were significantly associated with malnutrition, hypoalbuminaemia, erythropoietin resistance and morbidity and mortality for cardiovascular disease [16,17]. There is a considerable inter-individual variability in the degree of systemic inflammatory activation because the cytokine expression and production is, in part, genetically determined. We evaluated the relative influence of a specific IFN-γ polymorphism on the 2-year CRP response in a group of chronic haemodialysis patients. We found that the presence of the +874 A/A IFN-γ polymorphism, or the IFN-γ 'low-producer' genotype, was associated with the greatest preventive effect on CRP elevation. In these IFN-γ 'low-producer' patients the frequency of abnormally elevated CRP over 2 years averaged only 37%, whereas in the 'high and intermediate-producer' groups this feature was similar to that of the entire haemodialysis population, i.e. 57%.

We also studied the effects of IFN-γ polymorphism on gene transcriptions of several pro- and anti-inflammatory cytokines including IFN-γ. Expression of cytokine genes has been evaluated at the level of individual cytokine mRNA content in circulating blood cells because cytokine serum levels do not accurately reflect rate of expression during haemodialysis. In the IFN-γ high- and intermediate-producer genotype patients mean mRNA levels of IFN-γ were 27 and 14 times greater than in the low-producer patients. IFN-γ is a principal mediator of innate as well as adaptive immunity [4,5]. In addition to the pivotal role of IFN-γ in host defence, its excessive release has been associated with chronic inflammatory conditions, including haemodialysis [4]. Among the biological activities of IFN-γ, activation of macrophages is of key importance. Accordingly, IFN-γ up-regulates a variety of pro-inflammatory mediators including IL-6 [18]. In fact, our IFN-γ 'low-producer' patients exhibited parallel reductions of mRNA levels of both IFN-γ and IL-6. This suggests that the effect of the IFN-γ 'low producer' genotype on frequency of CRP elevation could have been mediated, at least in part, by modulation of IL-6 expression [18].

The rapid kinetics of CRP metabolism appears to be closely parallel to the degree of inflammation. Nonetheless, even in periods free of clinical events, microinflammation and CRP levels fluctuate in time, in haemodialysis patients [3]. In order to reduce longitudinal variability within subjects, we have monitored serum CRP level every month in a cohort of clinically stable uraemic patients on haemodialysis that underwent prospective follow-up for 2 years. The overall level of systemic inflammation was then expressed as fraction of altered CRP values over the total number of determinations. The percentage

of altered CRP individual value exhibited a great inter-individual variability and the average value of this indicator of overall 2-year inflammatory response was 54% (the median was equal to 53%) with a coefficient of variation of 60%. Seven of the 102 patients followed in this study always exhibited normal serum CRP concentrations during the 2-year observation, whereas serum CRP was always found abnormal in 13 of the patients even in the absence of intercurrent infections. Our results demonstrate that a significant portion of such variability was explained by IFN-γ polymorphisms.

Numerous studies have shown that elevated levels of CRP are associated with increased cardiovascular mortality [16]. It remains to be determined whether this marker of inflammation actually has a causal relation with cardiovascular disease or simply reflect the underlying disease process. Nonetheless, evidence indicates that elevated CRP concentrations may be directly involved in the initiation and progression of atherosclerosis [16], particularly in patients with more frequent elevation of CRP such as those on haemodialysis [1–3]. In particular, recent studies have identified a synergistic interaction between CRP and IFN-γ on the pathogenesis of coronary atherosclerosis and its acute complications [19]. The present study underlines that IFN-γ gene polymorphism is one determinant of CRP levels in haemodialysis patients. We may predict therefore that IFN-γ gene polymorphism could also influence cardiovascular risk and mortality in these patients.

The annual mortality rate for the haemodialysis patients was 9.8%. There was no significant difference in mortality between patients with different IFN-γ genotypes. Nonetheless, the annual mortality rate tended to be lower in the patients with the 'low-producer' IFN-γ genotype (7.6%) than in the patients with the 'high-producer' IFN-γ genotype (13.6%). In our study the observation period was too short and the number of patients was insufficient to accurately assess the effect of IFN-γ genotypes on mortality rate. A further study with an appropriate experimental design will be needed to test the hypothesis that the 'low-producer' IFN-γ ±874 A/A genotype is associated with a lower risk of cardiovascular and/or all-cause mortality in haemodialysis patients.

Recent studies have clearly established that IL-10 genotype strongly influences the CRP variation range [11,20]. By limiting the inflammatory activation in end-stage renal disease patients, the 'high-producer' IL-10 genotype is associated with a lower risk of cardiovascular disease and even mortality [11,20]. Our study suggests that, in addition to the 'high-producer' IL-10 genotype, a 'low-producer' IFN-γ genotype may downregulate the inflammatory activation in dialysis patients and, possibly, cardiovascular risk.

In conclusion, we have shown that the inter-individual variability in the degree of systemic inflammatory response activation exhibited by haemodialysis patients is in part explained by polymorphisms of the IFN-γ gene, whereas the IFN-γ 'low-producer' genotype was associated with the greatest preventive effect on CRP elevation. These results suggest that patients with IFN-γ 'low-producer' genotype may be relatively protected from the risk of developing cachaexia and cardiovascular disease associated to the uraemic syndrome. In addition, we may speculate that the treatment of severe inflammation in haemodialysis patients should be also targeted towards downregulation of the IFN-γ gene.

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Conflict of interest statement. None declared.

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