Original Article

Polymorphisms in IFN-γ, TNF-α and IL-10 in Patients on Maintenance Haemodialysis

(haemodialysis / cytokine polymorphism / IL-10 / TNF-α / IFN-γ)

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Abstract. The dysbalance in the expression of pro-inflammatory and anti-inflammatory cytokines, which is partially genetically determined, might have essential impact on the clinical outcome and survival of haemodialysed (HD) patients. A total of 500 HD patients and 500 healthy controls were genotyped for three single-nucleotide polymorphisms (SNPs: *TNFA* -308G/A, *IL10* -1082G/A, *IFNG* +874A/T). To detect the SNPs' impact on clinical outcome and survival, the HD population was divided into two subgroups depending on the length of HD therapy. The genotypes and phenotypes were correlated with two years followed up laboratory parameters and survival of HD patients. The one-year HD departed patients exhibited significantly higher age (P = 0.0167), C-reactive protein (P = 0.0012), lower nutritional (body mass index, P = 0.0168; dry weight, P = 0.0207; albumin, P = 0.005; triglycerides, P = 0.0174), haematological (red blood cells count, P = 0.0210; haemoglobin, P = 0.0159; haematocrit, P = 0.0368) and HD efficacy parameters (Kt/V, P = 0.0273) compared to long-term HD survivors. Both HD and control population showed similar genotype distribution except for higher occurrence of *TNFA* A/A homozygotes in healthy controls (P = 0.008). There were no differences in both genotypes and phenotypes in HD subgroups because of the low number of patients in one-year HD departed patients. Neither genotype nor phenotype had an impact on patients' survival. From our results we cannot infer that the promoter region SNPs of immune system response-regulating cytokines IL-10, TNF-α and IFN-γ have a major impact on clinical outcome of patients on maintenance haemodialysis.

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Abbreviations: BMI – body mass index, CKD – chronic kidney disease, CRP – C-reactive protein, CVD – cardiovascular disease, ESRD – end-stage renal disease, HD – haemodialysis, IFN-γ – interferon γ, IL-10 – interleukin 10, RBC – red blood cell, SNP – single-nucleotide polymorphism, TNF-α – tumour necrosis factor α, URR – urea reduction ratio, WBC – white blood cells.

Introduction

Despite undoubted improvements in haemodialysation techniques, the morbidity and mortality of patients in end-stage renal disease (ESRD) remain unchanged. Chronic inflammation, malnutrition, anaemia and cardiovascular disease (CVD) are the main independent factors of long-term morbidity and mortality of haemodialysed (HD) patients.

ESRD is characterized by both impaired humoral and cellular immunity and permanent activation of the immune system (Daichou et al., 1999; Ando et al. 2005; Nairn et al., 2006). The circulating monocytes produce large amounts of pro-inflammatory cytokines followed by anti-inflammatory interleukin 10 (IL-10), which limits the inflammatory activation after pathogen elimination (Girndt et al., 2003). Already in early stages of chronic kidney disease (CKD 3 and 4), pro-inflammatory cytokines directly accelerate atherosclerosis because of affecting the endothelium function (Menon et al., 2005; Honda et al., 2006; Stenvinkel, 2006a). Persistent inflammatory state deteriorates nutritional state, suppresses bone marrow stem cells giving rise to anaemia, and shares essentially in erythropoietin therapy resistance that correlates with poor outcome of dialysed patients (Cooper et al., 2003; Kalantar-Zadeh et al., 2003; Stenvinkel, 2006b; de Francisco et al., 2009).

The pivotal role in native host defence is played by interferon γ (IFN-γ), which directly stimulates the inflammatory response through activation of inflammatory cells, transcriptional factor NF-κB and by increasing production of pro-inflammatory cytokines, mainly tumour necrosis factor α (TNF-α) and IL-6 (Biolo et al., 2006). TNF-α regulates both specific and non-specific immunity, stimulates protein catabolism and mediates

endothelial dysfunction strongly associated with CVD in ESRD (Balakrishnan et al., 2004). IL-10 is the main regulator of the adequate inflammatory response. In ESRD, the individuals with the ability to sufficiently enhance production of IL-10 better control both uraemia-associated state and infection, and they suffer less from coronary artery disease (Girndt, 2002; Seyrek et al., 2005; Girndt et al., 2006; Litjens et al., 2008). The promoter gene region polymorphisms of TNF-α, IFN-γ, IL-10 affecting transcriptional activity results in high-, intermediate- and low-producing phenotypes with essential biological impacts. The IL-10 high-producers show better immune response, infection control and lower risk of cardiovascular death (Girndt et al., 2002). The up-regulation of TNF-α, observed in progressive renal disease as the response to glomerular injury, is associated with metabolic syndrome and CVD (Ortiz et al., 1995). The IFN-γ low-producers exhibit a preventive effect on C-reactive protein (CRP) elevation, and the reduction of mRNA levels of both IFN-γ and IL-6 thus may be relatively protected from CVD because of direct CRP involvement in atherosclerosis (Biolo et al., 2006).

Because clinical experience shows inter-individual differences in the level of immune system activation, it might be suggested that the individual ability to enhance or depress the pro- and anti-inflammatory cytokine production is partially genetically determined. In the presented study we assessed the polymorphisms of three major immunoregulatory cytokines TNF-α, IFN-γ, IL-10 in haemodialysed and representative population to determine the possible genetic influence on the disease clinical course in patients on maintenance haemodialysis.

Material and Methods

Subjects

The 500 adults in ESRD treated with HD taken from "GenDeMIA" population (Hubacek et al., 2009), and 500 age- and sex-matched controls taken from "MONICA" population representing a cohort of the selected 1 % Czech population sample without renal disorder (Multinational monitoring of trends and determinants in cardiovascular diseases: MONICA Project) were enrolled into the study. The laboratory parameters (CRP, creatinine, urea, lipids, BMI, albumin, WBC, RBC, haemoglobin, haematocrit, transferin, iron, URR, CaP product) of HD patients were prospectively followed up for two years by means of specially created electronic questionnaire. The primary measurements were made at study initiation and updated every three months.

Unfortunately, based on available clinical data, the haemodialysed patients could not have been further stratified in accordance with their exact cause of kidney disease resulting in end-stage renal failure. Thus, to analyse the genetic influence on clinical outcome and survival, we divided the HD population into two subgroups depending on the duration of HD therapy: departed patients within a year on HD (N = 32) and long-term survivors (N = 468).

DNA extraction and analysis

DNA was extracted from 5 ml whole uncoagulated (EDTA) blood samples by the standard salting-out method (Miller et al., 1988). DNA variants (all single-nucleotide polymorphisms – SNPs) of *IL10* (-1082G/A), *IFNG* (+874A/T) and *TNFA* (-308G/A) genes were analysed using PCR-RFLP-based methods in accordance with the procedures presented in detail previously (Brabcova et al., 2007). After cytokine genotype determination, the phenotypes for IL-10, IFN-γ and TNF-α were each assigned. These production phenotypes were based on previously published *in vitro* transfection studies using constructs of the relevant alleles, studies on whole blood and on PBMC cultures stimulated with endotoxin, and *in vivo* studies measuring plasma levels of the relevant cytokines (Balakrishnan et al., 2004). The low-, intermediate- and high-producer phenotype was assigned: IL-10, position -1082: low-producer genotype (A/A), intermediate-producer genotype (G/A), high--producer genotype; TNF-α, position -308: low-producer genotype (G/G), high-producer genotype (A/A and G/A); IFN-γ, position +874: low-producer genotype (A/A), intermediate-producer genotype (A/T), high-producer genotype (T/T).

Statistical analysis

Statistical analysis was performed using the χ^2 test, *t*-test or Mann-Whitney test. Values are given as mean ± SD. P value < 0.05 was considered to be significant.

Results

Demographic characteristics during two-year follow-up

The HD patients' average demographic characteristics during the two-year follow-up period are summarized in Table 1. The patients who died within the first year of HD exhibited significantly higher age (P = 0.0167), CRP (P = 0.0012), lower nutritional (BMI, P = 0.0168; dry weight, P = 0.0207; albumin, P = 0.005; triglycerides, P = 0.0174), haematological (red blood cells count, P = 0.0210; haemoglobin, P = 0.0159; haematocrit, P = 0.0368) and HD efficacy parameters (Kt/V, P = 0.0273) compared to long-term HD survivors.

Genotype and phenotype distribution

The distribution of *IL10* and *IFNG* gene polymorphisms in both HD and control "MONICA" populations was similar. More A/A homozygotes in the *TNFA* gene occurred in control population compared to HD population (P = 0.008) (Table 2).

Between HD groups we observed no significant differences in genotype and phenotype distribution. The patients who died within the first year after HD therapy

Table 1. Average clinical and laboratory characteristics of HD patients during two-year follow-up

	Enrolled HD patients	Departed patients within the 1st year on HD	Long-term HD survivors	P value
N	500	32	468	
Age (years)	65.4 ± 13.1	70.8 ± 12.7	65.1 ± 13.1	0.0167
BMI (kg/m2)	26.4 ± 5.1	24.6 ± 7.1	26.3 ± 4.9	0.0168
Dry weight (kg)	73.2 ± 15.4	65.7 ± 17.6	72.8 ± 15.1	0.0207
CRP (mg/l)	12.0 ± 19.0	35.7 ± 35.4	12.7 ± 16.7	0.0012
Cholesterol (mmol/l)	4.59 ± 1.10	4.31 ± 0.85	4.62 ± 0.99	ns
Triglycerides (mmol/l)	2.32 ± 1.75	1.62 ± 0.69	2.24 ± 1.42	0.0174
Albumin (g/l)	39.3 ± 4.3	37.0 ± 3.6	39.0 ± 3.6	0.005
Urea (mmol/l)	20.6 ± 6.5	18.7 ± 7.3	20.0 ± 4.9	ns
WBC (109/l)	7.2 ± 2.2	7.7 ± 2.3	7.2 ± 1.9	ns
RBC (1012/l)	3.6 ± 0.5	3.3 ± 0.6	3.6 ± 0.4	0.021
Haemoglobin (g/l)	112.0 ± 18.0	105.0 ± 25.0	114.0 ± 17.4	0.0159
Haematocrit	0.35 ± 0.06	0.33 ± 0.05	0.35 ± 0.04	0.0368
Transferrin (mmol/l)	2.56 ± 4.10	1.69 ± 0.40	2.83 ± 4.30	ns
Fe (umol/l)	12.7 ± 6.1	13.7 ± 8.5	12.5 ± 4.2	ns
CaxP product	4.23 ± 1.42	3.97 ± 1.37	4.34 ± 1.09	0.0528
Kt/V	1.49 ± 0.33	1.36 ± 0.26	1.49 ± 0.29	0.0273
URR	69.7 ± 17.8	70.5 ± 8.6	71.1 ± 10.6	ns

Table 2. Genotype distribution of IL10, TNFA and IFNG in "HD" and control "MONICA" population

Genotype	MONICA population	HD population	P value
IL10: -1082 (N/%)			
A/A	90/18.0	79/16.1	
G/A	269/53.8	267/54.2	0.759
G/G	141/28.2	146/29.7	
IFNG: +874 (%)			
A/A	100/20.0	99/20.5	
A/T	246/49.8	254/52.7	0.613
T/T	149/30.2	129/26.8	
TNFA: -308 (%)			
A/A	17/3.5*	3/0.7*	
G/A	143/29.2	120/24.8	0.008
G/G	330/67.3	360/74.5	

initiation only exhibited a tendency towards lower proportion of IL-10 high-producer and TNF-α low-producer genotype and higher proportion of both TNF-α and IFN-γ high-producer genotypes compared to long-term HD survivors, but without statistical significance (Table 3).

Relationship of cytokine polymorphisms, laboratory parameter follow-up, and survival

In our sample of HD patients, all three analysed polymorphisms exhibited only a tendency towards development of CRP, triglyceride, serum iron and calcium-phosphate product levels. Similarly, in IL-10 and TNF-α variants we observed a tendency towards BMI and dry weight development. The IFN-γ low producers showed higher BMI and cholesterol levels compared to both intermediate and high producers. Likewise, the TNF-α low producers exhibited higher leukocytes, red blood cells and haematocrit than high producers. None of the assessed genotype or phenotype had an impact on HD patients' survival.

Discussion

The clinical experience shows a substantial inter- and intra-individual variability in the manifestation of complications of HD therapy. Unrelated to dialysis procedure adequacy, ESRD is associated with persistent low-grade systemic inflammation that together with malnutrition and anaemia represents the main factors of morbidity

Table 3. Genotype and phenotype distribution of IL-10, TNF-α and IFN-γ in HD population

Cytokine	Genotype	Phenotype	Departed patients within the 1st year on HD	Long-term HD survivors	P value
IL-10	-1082 A/A (%)	low producer	15.63	15.93	ns
	-1082 G/A (%)	intermediate producer	62.50	52.33	ns
	-1082 G/G (%)	high producer	21.88	31.75	ns
IFN- γ	+874 A/A (%)	low producer	18.75	20.94	ns
	+874 A/T (%)	intermediate producer	43.75	54.20	ns
	+874 T/T (%)	high producer	37.50	24.89	ns
TNF- α	-308 A/A, G/A (%)	high producer	35.48	25.29	ns
	-308 G/G (%)	low producer	64.52	74.72	ns

and mortality of HD patients. The level of immune system imbalance with disturbance of the pro- and anti-inflammatory cytokine expression suggests being, in part, genetically determined. In the presented study we focused on the impact of polymorphism of three major immune response regulating cytokines, IL-10, IFN-γ, TNF-α, on the clinical outcome and survival of two years prospectively followed group of ESRD patients on maintenance HD. To determine the influence of IL-10, IFN-γ, TNF-α, the ESRD patients were divided into two subgroups depending on the length of HD treatment – patients who died already within the first year of therapy and patients who survived more than a year of therapy.

The enhanced inflammatory response of ESRD patients with the evidence of polarization of immune system towards the Th1 pattern is characterized by increased secretion of IFN-γ and TNF-α (Costa et al., 2008). The *IFNG* gene polymorphism is just one of CRP level determinants in HD patients. The high and intermediate (T/T, A/T) producer genotypes are associated with higher CRP levels compared to the low-producer genotype (A/A). Because of the synergistic interaction between CRP and IFN-γ, IFN-γ participates in the pathogenesis of atherosclerosis and CVD, respectively (Balakrishnan et al., 2004; Biolo et al., 2006; Perunicic-Pekovic et al., 2008). The TNF-α high-producer genotype (or the presence of -308A allele of *TNFA*) associated with high promoter activity results in enhanced TNF-α production and potentiates the susceptibility to chronic kidney disease compared to wild-type low-producer genotype (Ranganath et al., 2009). Increased amounts of TNF-α promote the pro-apoptotic actions of IFN-γ, which leads to early death of erythroid progenitor cells in the bone marrow and antagonizes the anti-apoptotic action of recombinant erythropoietin and erythropoietin itself (Stenvinkel, 2001, 2006b; Macdougall and Cooper, 2002; Mullarky et al., 2007). However, we have surprisingly found a significantly higher proportion of TNF-α high-producer A/A genotype in healthy controls in comparison with HD population (Table 2). IL-10 is secreted by regulatory T cells ($CD4^+/CD25^+$) and monocytes to control the inflammatory response adequacy. In ESRD, there are elevated plasma levels of IL-10 due to increased production by uraemic monocytes and decreased clearance through the kidneys (Stenvinkel et al., 2005). Because of the regulation of cholesterol metabolism and influence on CRP levels, the *IL10* genotype is regarded as risk factor for CVD in ESRD (Girndt et al., 2003; Loppnow et al., 2008; Han et al., 2009). The carriers of G allele at position -1082 in the promoter region show higher levels of IL-10, hence better immune response control.

The two-year followed nutritional, haematological and dialysis procedure efficacy findings in our HD population are in accordance with the observed survival. The patients who died within the first year after HD initiation exhibited significantly poorer nutritional (lower BMI, dry weight, albuminaemia, triglyceridaemia), haematological (more pronounced anaemia), inflammatory (higher CRP, leukocytosis) and dialysis efficacy (lower Kt/V) parameters compared to the long-term survival HD group (Table 1). The observed insufficient dialysis might be due to higher morbidity and significantly higher age of this group of patients. Compared with long-term HD therapy survivors, the first year HD therapy departed patients showed a tendency towards higher representation of TNF-α and IFN-γ high-producer and lower representation of IL-10 high- and TNF-α low-producer phenotypes corresponding with higher levels of markers of inflammation (CRP, WBC), of catabolic state (hypalbuminaemia, hypotriglyceridaemia) and of anaemia. Nevertheless, neither genotype nor phenotype distribution reached statistical significance because of the low number of individuals in this group of patients (Table 3). Similarly, we did not notice any significant influence of both genotypes and phenotypes of the analysed genes on patients' survival.

In conclusion, we cannot infer from our results that the promoter region polymorphisms of three major immunoregulating cytokines, TNF-α, IFN-γ and IL-10, have an important impact on the clinical outcome and survival of patients on maintenance haemodialysis.

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