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Cytokine Gene Polymorphisms are Associated with Serum Levels of Cardiovascular Risk Markers in Renal Transplant Recipients

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Abstract

Introduction

Cardiovascular disease represents the main cause of morbidity and mortality after renal transplantation. We have previously reported that functional cytokine gene polymorphisms affecting their expression and serum levels are candidates as potential cardiovascular risk factors in kidney transplant recipients. This study investigated the influence of cytokine production genotypes on the serum levels of the some well-assessed cardiovascular risk markers after renal transplant.

Methods

In a population of 477 renal transplant recipients we compared the serum levels of homocysteine, Lipoprotein(a) (Lpa), C-reactive protein (CRP), fibrinogen, LDL-cholesterol, tissue plasminogen activator (t-PA), monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), P-selectin and CD40 ligand (CD40L) between patients carrying different genotypes of the inflammatory cytokines TNF-α, IL-6, IL-10, TGF-β1, and IFN-γ.

Results

Significantly increased levels of CRP were found in TNF-α high producers compared to the low producers. IL-6 high producers displayed greater levels of VCAM-1 compared to the low producers. IL-10 high, intermediate and low producers displayed significant differences in homocysteine, fibrinogen and t-PA levels. The concentrations of VCAM-1, P-selectin and CD40L differed significantly in the TGF-β1 high, intermediate and low producers. The three producing genotypes of IFN-γ showed significant differences in the levels of homocysteine and MCP-1.

Conclusions

This work indicates that cytokine polymorphisms might represent cardiovascular risk markers in renal transplant.

Keywords: Kidney transplant; Cytokine polymorphisms; Cardiovascular disease; Cardiovascular risk markers

Abbreviations: ANOVA: Analysis of Variance; CD40L: CD40 Ligand; CRP: C-Reactive Protein; CVD: Cardiovascular Disease; IFN-γ: Interferon Gamma; IL-1: Interleukin-1; IL-6: Interleukin 6; IL-10: Interleukin 10; Lpa: Lipoprotein(a); MCP-1: Monocyte Chemoattractant Protein-1; ROS: Reactive Oxygen Species; TGF-β1: Transforming Growth Factor Beta 1; TNF-α: Tumor Necrosis Factor Alpha; t-PA: tissue Plasminogen Activator; VCAM-1: Vascular Cell Adhesion Molecule-1

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Introduction

Cardiovascular complications play are a major role in mortality and morbility of renal transplant recipients, and represent the leading cause of death with functioning graft [1-3].

The 25th US Renal Data System (USRDS) Annual Data Report containing data through 2011, shows that among recipients died with a functioning graft during 2007-2011, cardiovascular disease (CVD) was reported as the leading cause of death, accounting for 31% of these deaths, followed by infectious causes (19%) and malignancies (10%) [4]. The high prevalence of acronym CVD in kidney transplant recipients maybe due to the severe and progressive accumulation of atherogenic risk factors since the beginning of primary nephropathy to the period following transplantation [3-5]. It is known that the Framingham Heart Study equation underestimates the risk of cardiovascular complications after renal transplant owing to the considerable contribution given by non-traditional and uremia-related risk factors, above all chronic systemic inflammation [6-7]. Kiberd et al. proposed a revision of the Framingham risk score to improve the predictivity of cardiovascular outcomes in kidney transplant clinic [8]. In this regard, the time on dialysis, the prolonged anemia, the chronic immunosuppression are likely to trigger a combination of immunological responses, prothrombotic state, dysmetabolic alterations and inflammatory abnormalities which lead to a substantially increased cardiovascular risk in comparison to the general population. Although the process of atherogenesis has been formerly related to the accumulation of lipids within the artery wall, at present it is largely considered as an inflammatory disease. The contemporary concept indicates inflammation as the most prominent contributor for the development of CVD, involving both inflammatory cells and cytokines [9].

The balance between pro-inflammatory and anti-inflammatory cytokines in the atherosclerotic plaque microenvironment as well in the serum are important indicators of cardiovascular disorders [10]. Cytokine gene polymorphisms have been associated with high and low cytokine production and may modulate the magnitude of inflammatory responses and cardiovascular risk following transplantation [11-13]. In a previous case-control study on 798 renal transplant recipients, we have reported that the carriage of the genetically determined increased production of tumor necrosis factor alpha (TNF-α) and interleukin 10 (IL-10), respectively, as a risk factor and protection against CVD after renal transplant [13].

This study aims at investigating genotype/phenotype interactions in a population of Italian kidney transplant recipients (KTR), comparing across patients carrying different TNF-α, interleukin 6 (IL-6), IL-10, transforming growth factor beta 1 (TGF-β1), and interferon gamma (IFN-γ) genotypes the serum levels of the some well-assessed cardiovascular risk markers, namely homocysteine, Lipoprotein(a) (Lpa), C-reactive protein (CRP), fibrinogen, LDL-cholesterol, tissue plasminogen activator (t-PA), monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), P-selectin and CD40 ligand (CD40L).

Methods and Materials

Patients

The patient population was selected from a cohort of over 642 Italian KTR transplanted at our centre between January 2004 and June 2012.

Recipients with age younger than 18 years, pregnancy, diabetes or insulin resistance, sepsis, cancer or other diseases with fatal prognosis were excluded from the study.

Of the 477 selected patients (289 males, 188 females), the following demographic and clinical data were collected: sex, recipient and donor age, body weight, time on dialysis, cold ischemia time, HLA-mismatches, delayed graft function, serum creatinine at hospital discharge and at 1, 2, 3 and 4 years post-transplant, immunosuppressive therapy, primary nephropathy and presence of CVD.

CVD included ischemic heart disease, myocardial infarction, angina pectoris, ischemic cerebrovascular disease, ischemic stroke and other ischemic cerebrovascular diseases.

Coronary heart disease was defined according to the following criteria: (a) documented myocardial infarction, proven by an elevated creatine kinase level and electrocardiogram changes; (b) a clinical history of symptoms consistent with angina confirmed by a positive exercise tolerance test; (c) presence of significant stenosis (>50% luminal diameter) in at least one major coronary artery on coronary angiogram.

Cerebrovascular disease was indicated by the presence of at least one of the following conditions: (a) episode of cerebrovascular ischemia diagnosed by magnetic resonance angiography or computed tomography; (b) echo-Doppler-proven evidence of carotid artery stenosis > 50% for carotid vessels.

The diagnosis of peripheral vascular disease was established in the presence of one of the following: (a) intermittent claudication as indicated by previous bypass surgery or lower limb amputation; (b) angiographic or Doppler evidence of diffuse and severe peripheral atherosclerosis.

The protocol was approved by the Institution Ethics Committee and all subjects gave informed consent.

Genotype Analysis

The extraction of genomic DNA from EDTA-treated blood was performed using the GenomicPrep™ Blood DNA Isolation Kit (Amersham Biosciences, Piscataway, NJ, USA).

TNF-α/G-308A, IL-6/G-174C, IL-10/G-1082A, IL-10/C-819T, IL-10/C-592A, TGF-β1/L10P, TGF-β1/R25P, and IFN-γ/T+874A polymorphisms were determined by PCR-SSP through a commercially available kit (Cytokine Genotyping Tray, One Lambda, Inc. Canoga Park, CA, USA).

This methodology based on a cytokine genotyping tray identifies those genotypes related to the expression levels of each cytokine.

Amplifications were performed in Thermal Cycler Gene Amp PCR System 9700 (PE Applied Biosystems, Foster City, USA) according to the manufacturer's protocol. The PCR products were then run on a 2.5% agarose gel stained with ethidium bromide (Bio Whittacker Molecular Applications, Rockland, ME, USA) and the genotype determined by the banding pattern observed.

Serum Markers of Cardiovascular Risk

Blood samples were collected in Vacutainer 8.0 ml tubes (green top with beads clot activator) and sera were frozen at -70°C if not analyzed immediately. Homocysteine, Lpa, CRP, fibrinogen and LDL-cholesterol were determined using routine methods. The soluble forms of P-selectin, CD40L, t-PA, VCAM-1 and MCP-1 were measured by FlowCytomix assay (Bender MedSystems GmbH, Vienna, Austria) according to the manufacturer's recommendations.

Statistical Analysis

Deviations from Hardy-Weinberg equilibrium were analyzed using a χ^2 test. Demographic and laboratory values are reported as mean ± standard deviation.

Student's t-test, the Kolmogorov-Smirnov test, and Fisher's F-test were used to analyze the differences between high and low producers of TNF-α and IL-6 in the levels each of cardiovascular risk marker. High, intermediate and low producers of IL-10, TGF-β1, and IFN-γ were compared by one-way analysis of variance (ANOVA). The Tukey post-hoc test was then used for pairwise comparisons between high, intermediate and low producers of IL-10, TGF-β1, and IFN-γ. A p value below 0.05 was considered statistically significant. All statistical analyses were performed using SPSS, version 20.0.

Conclusions

Our work shows that KTR carrying different cytokine production genotypes display distinct patterns of some well-established serum markers of cardiovascular risk, namely homocysteine, Lipoprotein(a) (Lpa), C-reactive protein (CRP), fibrinogen, LDL-cholesterol, tissue plasminogen activator (t-PA), monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), P-selectin and CD40 ligand (CD40L).

Although further prospective studies on a larger population are needed to fully clarify the prognostic value of cytokine polymorphisms on CVD after renal transplant, this study seems to indicate that cardiovascular outcome in each patient is likely to be also considerably modulated by a genetic component.

This kind of approach may represent a valuable tool for the stratification of KTR according to their individual susceptibility to atherosclerosis and for the application of genotyping in the tailoring of therapeutic interventions.

Results

In this investigation, we genotyped 477 kidney recipients for TNF-α, IL-6, IL-10, TGF-β1 and IFN-γ polymorphisms. All polymorphisms were compatible with Hardy-Weinberg equilibrium (p>0.05, data not shown).

Demographic characteristics, biochemical, therapeutic and transplant-related parameters of the patient population are summarized in Table 1. The number and type of cardiovascular events are reported in Table 2. Graft failure occurred in 67 patients.

The assignment of cytokine production, based on previous in vitro and in vivo studies [14], was as follows: TNF-α position -308: high producer (AA or GA), low producer (GG); IL-6 position -174: high producer (GG or GC), low producer (CC); IL-10 positions -1082, -819 and -592: high producer (GCC/GCC), intermediate producer (GCC/ACC or GCC/ATA), low producer (ACC/ACC or ACC/ATA or ATA/ATA); TGF-β1 codon 10 and 25: high producer (TG/TG or CG/TG), intermediate producer (CC/TG or CG/CG or TG/TC), low producer (CG/CC or CC/CC or TC/TC or TC/CC); IFN-γ position +874: high producer (TT), intermediate producer (TA), low producer (AA).

Tables 3 and 4 show the results of the grouped comparisons of serum cardiovascular markers between high, intermediate (where possible) and low producers. No difference was found with regard to demographic characteristics, biochemical, therapeutic and transplant-related parameters between the various cytokine production categories (data not shown).

Sex	M: 289; F:188
Recipient age (years)	42.7 ± 12.9
Donor age (years)	43.5 ± 16.8
Body weight (kg)	65.6 ± 6.8
Time on dialysis (months)	30.8 ± 27.5
Cold ischemia time (hours)	16.7 ± 7.2
HLA A mismatches	1.29 ± 0.66
HLA B mismatches	1.28 ± 0.67
HLA DR mismatches	0.75 ± 0.64
Delayed graft function (days)	3.9 ± 5.4
Serum creatinine at hospital discharge (mg/dL)	1.31 ± 0.32
Serum creatinine at 1 year (mg/dL)	1.46 ± 0.39
Serum creatinine at 2 years (mg/dL)	1.54 ± 0.46
Serum creatinine at 3 years (mg/dL)	1.62 ± 0.49
Serum creatinine at 4 years (mg/dL)	1.64 ± 0.64
Immunosuppressive therapy	
–Steroids	295/477
–Cyclosporin A	240/477
–Azathioprine	81/477
–Mycophenolate mofetil	95/477
–Tacrolimus	133/477
–Sirolimus/Everolimus	26/477
Primary disease	
–Glomerulonephritis	135/477
–Polycistic kidney disease	105/477
–IgA nephropathy	41/477
–Interstitial nephritis	56/477
–Vascular nephropathy	53/477
–Hereditary nephropathy	34/477
–Not diagnosed	43/477

Table 1. Demographic characteristics, biochemical, therapeutic and transplant-related parameters of the patient population (n=477).

Cardiovascular events	***N***
Myocardial infarction	21
Stroke	7
Heart failure	15
Arrhythmia	11
Stenosis (>50%)	35
Angina pectoris	9
Peripheral artery disease	5
Transient ischemic attack	6
Total	*109*

Table 2. Cardiovascular events in the study cohort during follow-up.

Table 3. Serum levels of cardiovascular risk markers across genotypes of TNF-α and IL-6carried by renal transplant patients. Values are presented as mean ± standard deviation.

Cytokine	TNF-α			IL-6		
Producer genotype	**High (n=118)**	**Low (n=359)**	**p**	**High (n=428)**	**Low (n=49)**	**p**
Homocysteine, µmol/L	18.7 ± 5.4	20.0 ± 7.1	ns	19.3 ± 6.5	21.2 ± 9.4	ns
Lpa, mg/dL	28.5 ± 32.0	25.6 ± 40.3	ns	25.7 ± 40.1	29.1 ± 30.4	ns
CRP, mg/dL	1.02 ± 1.51	0.51 ± 0.50	<0.001	0.63 ± 0.87	0.47 ± 0.31	ns
Fibrinogen, mg/dL	318.3 ± 101.7	323.0 ± 61.9	ns	319.2 ± 73.8	342.4 ± 50.3	ns
LDL-C, mg/dL	110.7 ± 29.5	117.9 ± 40.7	ns	117.3 ± 39.4	105.3 ± 24.2	ns
t-PA, ng/mL	5.55 ± 2.77	6.25 ± 5.31	ns	6.16 ± 5.01	5.30 ± 2.37	ns
MCP-1, pg/mL	128.7 ± 108.2	237.4 ± 558.5	ns	214.6 ± 518.8	236.4 ± 248.7	ns
VCAM-1, ng/mL	1620.4 ± 1394.5	1456.6 ± 1417.3	ns	1554.9 ± 1429.9	790.7 ± 948.5	0.027
P-selectin, ng/mL	139.1 ± 224.8	132.2 ± 204.9	ns	137.4 ± 217.7	91.9 ± 6.8	ns
CD40L, ng/mL	17.33 ± 12.21	16.81 ± 37.52	ns	16.9 ± 34.8	16.7 ± 10.9	ns

CD40L, CD40 ligand; CRP, C-reactive protein; IL-6, interleukin 6; IL-10, LDL-C, LDL cholesterol; Lpa, lipoprotein a; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor alpha; t-PA, tissue plasminogen activator; VCAM-1, vascular cell adhesion molecule-1

Table 4. Serum levels of cardiovascular risk markers across genotypes of IL-10, TGF-β1 and IFN-γ carried by renal transplant patients. Values are presented as mean ± standard deviation.

Cytokine	IL-10				TGF-β1				IFN-γ			
Producer genotype	**High (n=68)**	**Intermediate (n=249)**	**Low (n=160)**	**p**	**High (n=326)**	**Intermediate (n=121)**	**Low (n=30)**	**p**	**High (n=130)**	**Intermediate (n=214)**	**Low n=133)**	**p**
Homocysteine, µmol/L	15.6 ± 5.4	16.1 ± 5.6	22.8 ± 6.4	<0.001	19.9 ± 7.7	18.4 ± 4.5	21.2 ± 5.7	ns	23.9 ± 8.1	18.7 ± 6.1	16.0 ± 5.7	<0.001
Lpa, mg/dL	19.7 ± 33.2	28.3 ± 46.6	26.2 ± 29.3	ns	27.0 ± 42.7	24.0 ± 31.3	25.7 ± 32.5	ns	27.6 ± 30.7	28.7 ± 50.3	20.0 ± 23.3	ns
CRP, mg/dL	0.33 ± 0.07	0.41 ± 1.03	0.37 ± 0.67	ns	0.59 ± 0.89	0.70 ± 0.78	0.47 ± 0.35	ns	0.33 ± 0.09	0.48 ± 1.14	0.31 ± 0.41	ns
Fibrinogen, mg/dL	316.3 ± 65.9	315.9 ± 63.3	389.5 ± 58.8	<0.001	319.3 ± 75.2	327.5 ± 63.1	327.8 ± 70.4	ns	320.7 ± 58.7	313.2 ± 79.1	261.0 ± 71.0	ns
LDL-C, mg/dL	111.9 ± 35.0	116.8 ± 44.3	116.8 ± 28.3	ns	115.7 ± 35.6	118.6 ± 44.7	108.8 ± 39.4	ns	119.8 ± 35.3	120.7 ± 42.5	113.0 ± 30.9	ns
t-PA, ng/mL	5.67 ± 4.62	5.53 ± 4.33	7.98 ± 5.12	<0.001	6.57 ± 5.36	5.14 ± 3.51	4.58 ± 2.37	ns	5.37 ± 3.00	5.96 ± 5.48	5.06 ± 5.34	ns
MCP-1, pg/mL	359.3 ± 1092.5	191.3 ± 253.3	175.7 ± 195.0	ns	216.8 ± 572.7	244.1 ± 330.0	234.2 ± 151.9	ns	225.0 ± 129.5	203.2 ± 161.3	141.8 ± 196.0	<0.001
VCAM-1, ng/mL	1575.7 ± 1527.6	1569.8 ± 1358.7	1330.2 ± 1436.4	ns	1435.7 ± 1281.6	1389.8 ± 1346.6	2958.0 ± 2615.7	<0.001	1261.6 ± 1043.8	1296.2 ± 1202.5	1882.4 ± 1889.3	ns
P-selectin, ng/mL	90.7 ± 3.6	132.7 ± 207.5	156.7 ± 256.5	ns	137.8 ± 218.8	91.6 ± 7.2	325.5 ± 464.4	<0.001	125.0 ± 192.6	159.7 ± 268.5	1205.3 ± 17.0	ns
CD40L, ng/mL	14.0 ± 10.0	17.1 ± 34.3	18.1 ± 39.8	ns	20.2 ± 40.0	9.66 ± 8.56	11.5 ± 6.5	0.016	20.6 ± 43.6	16.6 ± 34.9	5.6 ± 9.9	ns

CD40L, CD40 ligand; CRP, C-reactive protein; IFN-γ, interferon gamma; IL-10, interleukin 10; LDL-C, LDL cholesterol; Lpa, lipoprotein a; MCP-1, monocyte chemoattractant protein-1; TGF-β1, transforming growth factor beta-1; t-PA, tissue plasminogen activator; VCAM-1, vascular cell adhesion molecule-1.

Significantly superior levels of CRP were found in TNF-α high producers compared to the low producers (CRP: 1.02 ± 1.51 mg/dL vs 0.51 ± 0.50 mg/dL, p<0.001).

IL-6 high producers displayed significantly higher levels of VCAM-1 compared to the low producers (VCAM-1: 1554.9 ± 1429.9 ng/mL vs 790.7 ± 948.5 ng/mL, p=0.027).

The ANOVA test run on IL-10 genotypes across high, intermediate and low producers revealed significant differences in homocysteine, fibrinogen and t-PA levels (homocysteine: 15.6 ± 5.4 μmol/L vs 16.1 ± 6.4 μmol/L vs 22.8 ± 6.4 μmol/L, p<0.001; fibrinogen: 316.3 ± 65.9 mg/dL vs 315.9 ± 63.3 mg/dL vs 389.5 ± 58.8 mg/dL, p<0.001; t-PA: 5.67 ± 4.62 ng/mL vs 5.53 ± 4.33 ng/mL vs 7.98 ± 5.12 ng/mL, p<0.001). Post-hoc pairwise comparisons highlighted that the serum levels of homocysteine, fibrinogen and t-PA were significantly lower in the carriers of the high and intermediate producer genotype as compared to the ones with the low producer genotype (p<0.05), with no differences between high and intermediate producers (p=ns).

The concentrations of VCAM-1, P-selectin and CD40L differed significantly in the TGF-β1 high, intermediate producers and low producers (VCAM: 1435.7 ± 1281.6 ng/mL vs 1389.8 ± 1346.6 ng/mL vs 2958.0 ± 2615.7 ng/mL, p<0.001; P-selectin: 137.8 ± 218.8 ng/mL vs 91.6 ± 7.2 ng/mL vs 325.5 ± 464.4 ng/mL, p<0.001; CD40L: 20.2 ± 40.0 ng/mL vs 9.66 ± 8.56 ng/mL vs 11.5 ± 6.5 ng/mL, p=0.016). The Tukey post-hoc test proved that VCAM-1 and P-selectin levels were significantly decreased in TGF-β1 high and intermediate producers in comparison with the low producers (p<0.05), but it did not differ between high and intermediate producers (p=ns). The serum concentrations of the soluble form of CD40L were significantly greater in TGF-β1 high producers compared to the other producing genotypes, with no difference between intermediate and low producers (p=ns).

Finally, the three producing genotypes of IFN-γ showed significant differences in the levels of homocysteine and MCP-1 (homocysteine: 23.9 ± 8.1 μmol/L vs 18.7 ± 6.1 μmol/L vs 16.0 ± 5.7 μmol/L, p<0.001; MCP-1: 225.0 ± 129.5 pg/mL vs 203.2 ± 161.3 pg/mL vs 141.8 ± 196.0 pg/mL, p<0.001). Post-hoc multiple comparisons using Tukey test highlighted a progressive decrease in homocysteine levels from IFN-γ high to intermediate producers (p<0.01) and from IFN-γ intermediate to low producers (p<0.01). Serum MCP-1 levels were significantly higher in patients carrying the high or intermediate genotype compared to those with the low producer genotype (p<0.01), with no differences between the high and intermediate producers (p=ns).

Discussion

Current evidence supports the conception of atherosclerosis as an inflammatory process [9,15-16] and has implicated cytokine activation as an important pathogenic mechanism of atherogenesis and plaque destabilization [15]. Several regions of genetic diversity have been described in various cytokine genes and they have been proven to be susceptibility markers for CVD in the general population [17-20], as well as in transplant recipients [11-13]. Most of the cytokine polymorphisms have been shown to influence transcription, translation and the levels of protein in the atherosclerotic plaque microenvironment as well in the plasma [21]. In this study, we speculated the hypothesis that specific inherited variations in cytokine genes might affect the magnitude of inflammatory responses after renal transplantation and predispose or conversely protect against atherosclerosis, depending on the balance between the levels of pro-inflammatory and anti-inflammatory cytokines. For this purpose we compared the serum levels of the some cardiovascular risk markers between patients carrying different producing genotypes.

There is increasing proof that the regulation of TNF-α is crucial in atherosclerotic plaque rupture, because it causes weakening of the fibrous cap [22-23]. The A allele of the TNF-α/G-308A polymorphism has been associated with increased production of TNF-α [24] and might modulate the susceptibility to CVD and other diseases characterized by high TNF-α expression [25-26]. Poor data are currently available concerning cardiovascular risk in renal transplant recipients. We have previously reported that the carriage of TNF-α high producer genotype was associated with a significantly increased cardiovascular risk in an Italian population of renal kidney allografts [13]. Here, in the comparison between the serum levels of cardiovascular risk markers, we found that the levels of CRP were significantly more elevated in the TNF-α high producers than in the low producers and this finding seems to support the hypothesis that the carriage of the A allele, associated with augmented transcription activity, might promote proinflammatory status and enhance the risk of cardiovascular events in KTR. Nevertheless, an increase in TNF- α levels has been proven to have a direct effect on CRP, because cytokines, such as interleukin-1 (IL-1) and TNF-α are able to stimulate the production of IL-6, which induces hepatic production of acute-phase proteins including CRP [9,27].

IL-6 is recognized as an important contributor to atherosclerosis. IL-6 transcripts are present in atherosclerotic arterial wall [28], and IL-6 mRNA is expressed in human aortic and iliac fibrous plaques, indicating a local production of this cytokine [29]. Our data revealed higher levels of the adhesion molecule VCAM-1 in those patients carrying the high producer genotype of IL-6. One possible explanation may lie in the cytokine-induced endothelial activation, which results in an upregulation of VCAM-1. It is known that the pro-inflammatory cytokines IL-1 and IL-6 are involved in SMC-leukocyte interaction. Dysregulated endothelial functions induced by an altered cytokine production may trigger cell accumulation, LDL accumulation, deposition of extracellular matrix, and local and systemic overproduction of adhesion molecules. Consequently, vascular smooth muscle cell-fostered inflammation might represent an essential component for development and acceleration of atherosclerosis [30].

IL-10 is the most potent downregulator of proinflammatory cytokines, and it has been proposed that its local production by macrophages in the atheromata may represent a possible protective mechanism against vascular injury [31-32]. IL-10 levels are genetically determined by the combination of three biallelic polymorphisms at positions -592, -819 and -1082 in the promoter region of the IL-10 gene, resulting in three levels of production, high, intermediate and low [33].

The effects of IL-10 genotypes in atherosclerosis are controversial. Some studies in the general population have failed to find associations between the presence of CVD and IL-10 genotype [27], although the A allele for IL-10/G-1082A polymorphism, associated with lower IL-10 production, was found to be predictive of a higher cardiovascular morbidity in dialysis patients [34-35]. This difference may be explicated by the more evident influence of the IL-10 genotype in hemodialysis patients than in the general population, owing to the prolonged exposure to high-level inflammation. In our transplanted patients we observed significantly lower concentrations of homocysteine, fibrinogen and t-PA in IL-10 high and intermediate producer genotypes compared to the low producers. Although the pathogenetic link between IL-10 and hyperhomocysteinemia remains to be fully understood, a probable explanation is the capacity of IL-10 to suppress interferon production via the activation of STAT group transcription factors [36-37]. It is likely that IL-10 high/intermediate producers might be more efficient at downregulating the synthesis of IFN-γ, a potent trigger for the formation and release of reactive oxygen species (ROS) responsible for the depletion of antioxidants like vitamin C and E and glutathione. The subsequent chronic oxidative status may also explain the irreversible oxidation of folic acid and vitamin B12,

which are essential cofactors in homocysteine-methionine metabolism [38]. In the view of hyperhomocysteinemia as a consequence of immune activation, our data seem to substantiate the hypothesis that IL-10 high/intermediate producer genotype carriers might better control the elevation of serum homocysteine, the magnitude of inflammatory responses and the risk of atherogenetic complications after renal transplant. This assumption also seems to be confirmed by the significantly increased homocysteinemia detected in IFN-γ high producers in comparison with the low or intermediate producing genotypes. Our finding of the lower fibrinogen levels in IL-10 high/intermediate producers is consistent with previous studies demonstrating that the anti-inflammatory cytokines IL-4, IL-10 and IL-13 dose-dependently down regulate the synthesis of fibrinogen, with a resulting protective effect against vascular injury leading to atherosclerosis [39]. The link between IL-10 and coagulation cascade, although not fully elucidated, seems to be also suggested by the lower circulating t-PA in IL-10 high and intermediate producers, in agreement with the conception that IL-10 increased synthesis might have not only anti-inflammatory effects but also the ability to enhance endogenous fibrinolytic mechanisms. In a model of human endotoxemia focused on IL-10 in LPS-induced activation of the hemostatic mechanisms in vivo, it has been shown that the administration of recombinant human IL-10 before LPS challenge is able to limit LPS-induced activation, modulate the fibrinolytic system and reduce t-PA, plasmin-alpha2-antiplasmin complexes and D-dimer [40]. The effect of IL-10 on the regulation of the fibrinolytic system was also confirmed by Okada et al. in a successive study on a mouse ischemia/reperfusion model, where it was reported that IL-10 null mice had a poorer postischemic lung function and survival after I/R compared with IL-10 (+/+) mice. Recombinant IL-10 given to IL-10 (-/-) mice normalized the PAI/tissue-type plasminogen activator ratio, reduced pulmonary vascular fibrin deposition, and rescued mice from lung injury [41].

TGF-β1 is a pleiotropic cytokine, with a key role in the inhibition of atherogenesis [42,43]. Consistent with findings in non-uremic subjects [44], in a study by our group on 155 hemodialysis patients, we have previously demonstrated that decreased levels of TGF-β1 may predispose hemodialysis patients to CVD, and that a 1 ng/mL reduction in TGF-β1 serum concentration was associated with a 9% increase in the risk of cardiovascular mortality [45]. The production of the biologically active form of TGF-β1 is under genetic control and is predominantly related to L10P and R25P polymorphisms mapping in the first exon of TGF-β1 gene. The allele combination of the two polymorphisms generates different production levels [40] which have been implicated in the development of atherosclerosis in the general population [41], in hemodialysis patients [42] and kidney recipients [8].

In our patients the potential influence of the TGF-β1 genotype on cardiovascular risk after renal transplant seems to be supported by the detection of significantly increased levels of VCAM-1, P-selectin and CD40L in the TGF-β1 low producers compared to the other genotypes. Some experimental models have shown a local hyperexpression of VCAM-1, P-selectin and CD40L on the vascular endothelium of vulnerable plaque and a rise in the circulating soluble forms [49-50]. Since the TGF-β1 plays a crucial role in plaque stability, it can be hypothesized that the KTR carrying the low producer genotype might be more prone to develop cardiovascular events.

The role of IFN-γ in atherosclerosis remains to be clarified. It has been proposed that IFN-γ promotes the formation and release of reactive oxygen species (ROS), in concert with other pro-inflammatory cytokines, playing a central role in plaque formation and atherogenesis [38]. Limited data are available on the influence of IFN-γ genotype on cardiovascular risk [51-52]. Manginas et al. reported that on a Greek population of patients with the clinical presentation of coronary artery disease the low producing genotype of IFN-γ was more frequent in patients with stable angina compared to those with unstable angina or nonfatal myocardial infarction [52]. The influence of IFN-γ genotype on cardiovascular outcome in organ transplant recipients is debated. Densem et al. IFN-γ reported no association between a dinucleotide repeat in the first intron of the IFN-γ gene related to IFN-γ production and the risk of coronary vasculopathy after cardiac transplantation [53]. However IFN-γ has an essential role in the growth and function of T cells and macrophages, free radical formation, adhesion molecule expression, which are important processes in cardiovascular risk. We found that a higher genetic production of IFN-γ resulted in an increase in the levels of homocysteine and MCP-1. Hyperhomocysteinemia results from several genetic and non-genetic causes, including B-vitamin status. It is well known that macrophages stimulated by TH1-type cytokine IFN-γ determine the formation of ROS, able to oxidize antioxidants, lipoproteins and oxidation-sensitive B-vitamins, essential for homocysteine-methionine conversion [38]. Our data seem to suggest that high levels of IFN-γ might be implicated in the development of hyperhomocysteinemia, thus playing a major role in atherosclerosis and cardiovascular risk.

MCP-1, a member of the CC chemokine family, has been proven to be highly expressed in human atherosclerotic lesions [54]. A possible underlying mechanisms for the development and progression of atherosclerosis is the recruitment of inflammatory cells in the intima triggered by local production of chemokines and chemokine receptors [55]. In our population of KTR, the IFN-γ high or intermediate producers displayed significantly higher serum levels of MCP-1 compared to those with the low producer genotype. In animal models and humans, IFN-γ has been proven to have a direct effect on the production of chemokines from macrophages, including IL-8 and MCP-1 [55-56]. Bauermeister et al. showed that in cultured peritoneal macrophages from CAPD patients, addition of IFN-gamma resulted in a significant rise in MCP-1 synthesis [57]. Consistent with our data showing an increase of MCP-1 levels in those patients with a higher genetic production of IFN-γ, it is hypothesizable that IFN-γ produced by T lymphocytes might contribute to the cytokine network in regulating local inflammatory response and macrophage activation and differentiation, also inducing the release of MCP-1 from these cells.

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