Influence of Cytotoxic T-Lymphocyte Antigen-4 Polymorphisms on Acute Rejection Onset of Cadaveric Renal Transplants

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ABSTRACT

We retrospectively examined in cadaveric renal transplants the association between acute rejection episodes (ARE) and single nucleotide polymorphisms (SNPs) localized in the cytotoxic T-lymphocyte antigen (CTLA)-4 promoter, −1147T/C and −318C/T, in exon 1 +49A/G and within the 3′ untranslated region (UTR) CT60G/A. Each one of these SNPs may influence the cell surface expression of the CTLA-4 molecule. Seventy-two cadaveric renal transplant recipients with at least 6 month's follow-up were genotyped for CTLA-4 dimorphisms using direct sequencing of specific polymerase chain reaction products. Allele frequencies in both groups of patients with or without acute rejection (ARE and non-ARE) did not show significant differences in various nucleotide positions. At the level of genotype frequency we first noted a positive association to acute rejection of G/G genotypes (ARE af = 14.7%, non-ARE af = 5.9%) for the +49 (cod. 17), which was associated with decreased expression of the CTLA-4 full-length molecule. In contrast, the AG genotype seemed to be protective (61.8% vs 32.4%, $P = .028$; odds ratio [OR] = 0.2961). Regarding the CT60G/A dimorphism, noteworthy was the identification of a significantly higher incidence of CT60 A/A genotype in ARE compared with non-ARE group (29.7% vs 8.6%; Yates $P = .035$; OR = 4.51). Such association of *protective* AA genotype with ARE, as observed also in autoimmunity, was associated with an increased level of sCTLA-4 induced by the polymorphism, which blocks B7−flCTLA-4 interactions, enhancing T-cell reactivity by preventing transduction of inhibitory signals. Considering the various polymorphic sites in the haplotype, we observed a significant increase in ARE among patients of the CTLA4 +49A/CT60A (HF = 51.5% vs 29.5%; $P = .014$; OR = 2.545) and a reduction among the +49A/CT60G (17.6% vs 33.8%; $P = .04$; OR = 0.4193) 2-loci haplotype, As regards the −1147/−318/+49/CT60 CTLA-4 4-loci haplotypes, we observed a significantly higher frequency of the CCAA haplotype in ARE patients comparison with those free of rejection (HF = 51.8% vs 31.1%, $P = .046$ OR = 2.363). These findings are consistent with those observed in allogeneic stem cell transplantation, wherein patients with CT60 AA showed a major incidence of graft-versus-host disease. An association of protective AA genotype with ARE, as observed also in autoimmunity was associated with an increased level of sCTLA-4 induced by this polymorphism, which blocking the B7−flCTLA-4 interaction, would enhance T-cell reactivity by preventing transduction of inhibitory signals.

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ORGAN TRANSPLANTATION is the treatment of choice for terminal organ failure. Nevertheless, prevention of rejection requires immunosuppression, which increase the risk of complications but poorly influences chronic rejection, which is the main cause of graft loss in the long term. The study of the genetic variations, the manipulation of receptor/ligand signals involved in the antigen presenting cell (APC)/T cell interactions and the availability of new biological drugs have created enormous expectations. The alloimmune response is regulated by molecules that show genetic variability (gene polymorphisms). Evaluation of individual characteristics in the future will allow better reaction of the immunosuppressive regimen, avoiding excessive or insufficient effects, the so-called "tailored medicine."

The balance between "positive" and "negative" signaling is believed to enable effective immune responses, maintaining immunologic tolerance and preventing autoimmunity. Receptors like the cytotoxic T-lymphocyte antigen (CTLA-4) and the programmed death-1 antigen transduce signals that inhibit lymphocyte activation. The glycoprotein CTLA-4 is expressed transiently on activated $CD4^+$ and $CD8^+$ T cells and constitutively on T-regulatory cells, on a few nonlymphoid normal cells, on a variety of neoplastic cells, and on mature dendritic cells (DC), influencing their maturation and antigen presentation. In acts as a ligand to induce interferion-γ production by DCs and to prevent T-cell response via a mechanism that involves tryptophan catabolism. CTLA-4 behaves as a negative regulator of activation, inhibiting T Helper (TH) 1 and TH2 cytokine production, cell cycle progression upon interaction with ligands CD80/CD86 expressed on APCs[1,2] and influencing differentiation of $CD4^+$ TH2 cells. Several autoimmune diseases[3] and outcomes of organ transplantations have been associated with polymorphisms in the CTLA gene. Some studies have implicated CTLA-4 in the regulation of transplantation tolerance.[4–7] The CTLA gene produces an mRNA transcript that codifies a transmembrane protein (flCTLA-4) and another one, a soluble form of CTLA-4 (sCTLA-4), both of which influence regulation of receptor activity.[3]

The current retrospective study examined the interactions of several CTLA-4 allelic variants in the promoter, exon 1 and 3′ UTR regions to assess their impacts on the occurrence of acute rejection episodes after cadaveric renal transplantation. Particularly, the promoter −1147T/C (rs16840252) and −318 C/T (rs5742909), the exon 1 +49A/G (rs231775) and the 3′ UTR CT60G/A (rs3087243) and rs11571319 single nucleotide polymorphisms (SNPs) were studied in cadaveric renal transplant recipients of Caucasoid Italian origin. Each one of these SNPs may influence the cell surface expression of the CTLA-4 molecule. The former affects CTLA-4 gene transcription at the level of the promoter, the +49A/G polymorphism results in an amino acid substitution Thr17A1a of the leader peptide, and CT60A/G determines the efficiency of splicing and production of sCTLA-4.

MATERIALS AND METHODS

Seventy-two cadaveric renal transplant recipients with at least 6 months' follow-up were genotyped for CTLA-4 dimorphisms. Written informed consent was obtained from all participants. The study protocol was approved by our Ethics Committee Organ allocation was based on human leukocyte antigen (HLA)-DR, HLA-B and HLA-A matching, which was assessed using serologic typing. Clinical data monitored during follow-up included transplant-related information: panel-reactive antibody pretransplant, acute rejection episodes, delayed graft function, chronic rejection, and post-transplant complications. All patients underwent induction therapy with steroids and basiliximab at low dosages followed by cyclosporine or tacrolimus and mycophenolate mofetil.

Graft function was monitored by serum creatinine concentrations and the presence of acute rejection episodes (ARE) was defined by deterioration of renal function, as evidenced by 30% increased creatinine level from baseline that was not attributable to other reasons and recovered after antirejection treatment with pulse steroids and/or a positive transplant biopsy.

SNP Genotyping

Peripheral blood from patients was collected in EDTA tubes. Genomic DNA was extracted by a column-based nucleic acid purification method (Qlamp DNA Blood Midi kit), according to the manufactures' protocol. SNP polymorphisms were analyzed by the sequence-based typing (SBT) technique using primers already described in literature (−1147T/C[8] and −318C/T[9] in promoter; +49 Thr17Ala in exon 1[9] and CT60 A/G in 3′ UTR region of exon 4[10]). Polymerase chain reaction products were purified using QIAQuick spin columns (Qiagen) and sequence reactions (volume 25 uL) were performed using BigDye terminators chemistry v.3.1 and 1.1 (Applied Biosystems) and processed in a Sequence Analyzer ABI3130.

Statistical Analysis

Each polymorphism was tested for Hardy-Weinberg equilibrium via a 2-degree of freedom chi-square goodness of fit using the Guo and Thomson exact test. Associations with clinical outcomes were calculated using allelic and genotypic frequencies. Differences in genotype frequencies between groups were analyzed with reference to rejection status and/or antibody production using χ^2 test with Yates correction or by Fisher exact tests after Bonferroni correction, as appropriate. Haplotypes were calculated by the Expectation Maximum algorithm. Relative linkage disequilibrium between 2 alleles at 2 different loci evaluated[11] using Arlequin v.3.0 software was studied for associations with rejection and graft survival. Statistical analysis was performed using GraphPad Instat 3 software.

RESULTS

Allele frequencies among groups of patients with or without acute rejection (ARE and non-ARE) did not show significant differences in various nucleotide positions, −1147C/T (ARE C af = 82.8%, T af = 17.2%; non-ARE C af = 76.8%, T af = 23.2%), −318C/T (ARE C af = 93.1%, T af = 6.9%; non-ARE C af = 87.5%, T af = 12.5%), +49A/G (ARE A af = 69.1%, G af = 30.9%; non-ARE A af = 63.2%, G af = 36.8%) and CT60G/A (ARE A af = 50.0%, G af = 50.0%; non-ARE A af = 35.8%, G af = 64.3%, $P = .094$) or 3′ UTR A/G (ARE A af = 18.9%, G af = 81.8%, non-ARE A af = 24.1%, G af = 75.9%).

At the level of genotype frequency, we first noted for the +49 (cod.17) polymorphism a different distribution between the 2 groups (Table 1). Particularly, there was a positive association of ARE with G/G genotypes (ARE af = 14.7% vs non-ARE af = 5.9%), associated with decreased expression of the full-length CTLA-4 molecule, although not significantly. In contrast, the AG genotype seemed to be protective, because it was more frequent among the non-ARE group compared with the ARE cohort (61.8% vs 32.4%; $P = .028$; odds ratio [OR] = 0.2961).

Regarding the CT60G/A dimorphism, we identified a significantly higher incidence of CT60 A/A genotype among the ARE versus non-ARE group (29.7% vs 8.6%; Yates $P = .032$; OR = 4.51). In contrast, the other 3′ UTR dimorphism (rs11571319; G/A) did not show a correlation with acute rejection occurrence: ARE: AA af = 2.7%, GG 64.9%, AG 32.4% vs non-ARE: AA = 6.9%, GG 58.6%, AG 34.5% (Table 1). The genotype frequencies of all polymorphisms fit Hardy-Weinberg equilibrium in both patient groups.

Considering the various polymorphic sites in the haplotype, we observed a significant increase among ARE patients in the CTLA4 +49A/CT60A (HF = 51.5%, vs 29.5%; $P = .014$; OR = 2.545) and a reduction of the +49A/CT60G (17.6% vs 33.8%; $P = .04$; OR = 0.4193) 2-loci haplotype, while the haplotype CTLA-4 +49G-CT60A was displayed only by the non-ARE patient group (HF = 5.78%).

As regards the −1147/−318/+49/CT60 CTLA-4 4-loci haplotypes, we observed a significantly higher frequency of the CCAA haplotype in ARE compared with patients without rejection (HF = 51.8% vs 31.1%; $P = .046$; OR = 2.363; Table 2).

The pairwise linkage disequilibrium analysis for all pairs of loci identified a significant association between the −1147 and −318 promoter sites ($P = .0029$), between −1147 and CT60 ($P = .0019$) and particularly between +49 and CT60 sites (0.00000, $\chi^2 = 24.86734$), both in the ARE and the non-ARE groups.

DISCUSSION

This study indicated that the CTLA-4 +49A/G SNP, particularly the CT60G/A dimorphism, may have a role in genetic susceptibility to acute rejection episodes among Italian patients. As regards the first polymorphism, it is known that Ala17 variant (+49G) in the leader peptide of CTLA-4 was less efficiently glycosylated and transported to the cell surface. This substitution may influence the expression of the CTLA full-length molecule. The CTLA-4 +49G/G genotype shows an increased baseline proliferative capacity and a decreased response to CTLA-4 blockade compared with the A/G or A/A genotypes.[12] Our

Table 1. Cytotoxic T-Lymphocyte Antigen-4 Single Nucleotide Polymorphisms (SNP) Genotype and Allele Frequencies in Acute Rejection (ARE) and Non-Acute Rejection (non-ARE) Subjects

			ARE patients		Non-ARE patients			
Region	SNP	Genotype	Frequency	%	Frequency	%	*P* value	Odds ratio
Promoter	−1147, C/T	CC	20	69.0	16	57.1	NS	
	rs16840252	CT	8	27.6	11	39.3		
		TT	1	3.4	1	3.6		
		C		82.8		76.8		
		T		17.2		23.2		
	−318, C/T	CC	31	86.1	25	78.1	NS	
	rs5742909	CT	5	13.9	6	18.8		
		TT	0	0	1	3.1		
		C		93.1		87.5		
		T		6.9		12.5		
Exon 1	+49, A/G	AA	18	52.9	11	32.3		
Thr17Ala	rs231775	AG[a]	11	32.4	21	61.8	.028	0.2961
		GG	5	14.7	2	5.9	NS	
		A		69.1		63.2		
		G		30.9		36.8		
3′ UTR	CT60, G/A	AA[b]	11	29.7	3	8.6	.035	4.513
	rs3087243	AG	15	40.5	19	54.3		
		GG	11	29.7	13	37.1		
		A		50.0		35.7	.094	1.800
		G		50.0		64.3		
	G/A	AA	1	2.7	2	6.9	NS	
	rs11571319	AG	12	32.4	10	34.5		
		GG	24	64.9	17	58.6		
		A		18.9		24.1		
		G		81.8		75.9		

[a] *P* calculated by the comparison between AG and AA/GG genotypes.
[b] *P* calculated by the comparison between AA and AG/GG genotypes.

Table 2. Two- and 4-Loci Cytotoxic T-Lymphocyte Antigen (CTLA)-4 Haplotypes in Acute Rejection (ARE) and Non-Acute Rejection (non-ARE) Subjects

2-loci CTLA-4 Haplotypes		ARE patients ($n = 34$)		Non-ARE patients ($n = 34$)		
+49 A/G	CT60 G/A	Frequency	s.d.	Frequency	s.d.	*P* value
A	A	0.514706	0.055131	0.295055	0.057143	.0141, OR = 2.545
A	G	0.176471	0.042658	0.337298	0.064051	.048, OR = 0.4193
G	A	0	—	0.057887	0.037819	
G	G	0.308824	0.049683	0.309760	0.062848	

4-loci CTLA-4 Haplotypes				ARE patients ($n = 28$)		Non-ARE patients ($n = 24$)		
−1147 C/T	−318C/T	+49 A/G	CT60 A/G	Frequency	s.d.	Frequency	s.d.	*P* value
C	C	A	A	0.517857	0.071861	0.310951	0.073273	.046, OR = 2.363
C	C	G	G	0.303571	0.064624	0.373451	0.069646	
C	C	A	G	0	—	0.043216	0.028912	
C	C	G	A	0	—	0.022382	0.018958	
T	C	A	G	0.089286	0.038408	0.145833	0.056408	
T	C	G	G	0.017857	0.015233	0	—	
T	T	A	G	0.071429	0.038690	0.104167	0.044825	

OR, odds ratio; s.d., standard deviation.

results indicated a greater presence of the G/G variant, but above all, a protective effect of the AG genotype in ARE patients, as noted in other recent reports.[13,14] This association could be due to the reduced inhibitory function of the CTLA-4 molecule, which would predispose to an immune response toward a renal allograft. The effect of the +49A/A genotype on protection from acute rejection in transplanted patients was not evident among these patients who were transplanted with unrelated cadaveric donor, in contrast to that observed in living related donor recipients.[15]

In addition, the findings regarding CT60 polymorphisms seem to agree with those observed in allogeneic stem cell transplantation; patients with the CT60 AA genotype displayed a major incidence of severe graft-versus-host disease.[16] The association of the *protective* AA genotype with ARE, as also observed in autoimmunity, was associated with an increased level of soluble CTLA-4 induced by the polymorphism, which blocks the B7-flCTLA-4 interaction, enhancing T-cell reactivity by preventing transduction of inhibitory signals.

In addition, the contrasting effects of the CTLA-4+49A-CT60A ("predisposing") and CTLA-4+49A-CT60G haplotype ("protective") alleles, allow one to hypothesize a fundamental effect of the CT60 region on the onset of acute rejection. Finally, considering the 4 CTLA-4 polymorphic sites (−1147/−318/+49/CT60) in linkage, we noted a significant association of the CCAA haplotype with ARE, as already observed in chronic allograft dysfunction.[17] This finding indirectly confirms the *protective* effect of −318T variant on acute rejection, which in this study was 2 times less frequent in the ARE then the non-ARE cohort, as also cited in Wisniewski et al.[18] This effect has been previously explained by Ligers et al[19] and by Kouki et al.[12] Studies in nontransplanted subjects showed that a homozygous T allele at −318 position and/or homozygous A allele at +49 of exon 1 were associated with higher expression of the molecule in stimulated T cells and increased mRNA in unstimulated $CD4^+$ T cells.

The increased knowledge regarding variability and function of immunologic markers in the period after renal transplantation will be relevant to personalize immunosuppressive regimens, in order to reduce post-transplant complications and identify nonresponders or patients who could benefit from low-dose therapy. In fact, the immune activities of CTLA-4, including dephosphorylation of ZAP-70 tyrosine kinase modulate negatively the T cell receptor (TCR)-mediated signal transduction of T-cell activation upstream of the sites of immunosuppressive drug actions of cyclosporine or tacrolimus (inhibition of calcineurin action blocking interleukin-2 synthesis), or glucocorticoids (blocking nuclear factor-κb transcription factor).

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