

Chemokine Receptor Polymorphism and Risk of Acute Rejection in Human Renal Transplantation

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Abstract. Chemokines regulate the trafficking of leukocytes in immunity and inflammation and have been implicated in mouse models in acute cardiac and renal allograft rejection; however, their significance to human transplantation is not yet defined. The association of human chemokine receptor genetic variants, CCR5-Δ32, CCR5-59029-A/G, CCR2-V64I, CX3CR1-V249I, and CX3CR1-T280M, with outcome in 163 renal transplant recipients was examined here. Significant reductions were found in risk of acute renal transplant rejection in recipients who possessed the CCR2-64I allele (odds ratio [OR], 0.30; 95% confidence interval [CI], 0.12 to 0.78; $P =$

0.014) or who were homozygous for the 59029-A allele (OR, 0.37; 95% CI, 0.16 to 0.85; $P = 0.016$). There were no significant differences in the incidence of rejection among patients stratified as with or without CCR5-Δ32 or by the CX3CR1-V249I or CX3CR1-T280M genotypes. Adjustment for known risk factors for transplant rejection confirmed the univariate findings for possession of the CCR2-64I allele (OR, 0.20; $P = 0.032$) and homozygosity for the 59029-A allele (OR, 0.26; $P = 0.027$). It was concluded that the risk of acute rejection in renal transplantation is associated with genetic variation in the chemokine receptors CCR2 and CCR5.

Despite advances in immunosuppression and the overall medical care of renal transplant recipients, which have led to an improvement in allograft survival, chronic renal allograft rejection continues to be a major impediment to successful organ transplantation (1). Acute rejection is the single most important risk factor for developing chronic renal allograft rejection (2,3). Understanding the mechanisms that contribute to acute allograft rejection will be of great value for the development of improved antirejection strategies. Donor-recipient HLA mismatches, recipient race, donor age, and delayed graft function have been shown to be associated with rejection episodes (4). However, the currently understood risk factors explain only part of the variability in the longevity of the organ. Genetic polymorphism other than at the HLA locus is an attractive hypothesis for the explanation of the clinical heterogeneity of outcome in organ transplantation. Genetic variation could influence the function or expression of key immunoregulatory molecules that mediate transplant rejection.

In this regard, we have focused this study on chemokine receptors. Chemokines play a major role in the process by which leukocytes are recruited from the bloodstream into sites of inflammation, and several have been implicated in transplant rejection (5). The receptor CCR5 is specific for the proinflammatory chemokines, RANTES, MIP-1 α , and MIP-

1 β , and CCR2 and CX3CR1 bind MCP-1 and fractalkine, respectively (6). MCP-1, MIP-1 α , MIP-1 β , CCR2, and CCR5 have been shown to be markedly elevated in acutely rejecting human kidney transplants (7–9). Consistent with these findings is the fact that prolonged cardiac allograft survival has been achieved in CCR2 and CCR5 knockout mice (5), Met-RANTES, a CCR5 antagonist, can prolong renal allograft survival in an MHC-incompatible rat model (10), and treatment with an anti-CX3CR1 monoclonal antibody significantly prolonged cardiac allograft survival in mice (11).

To date, no data on the relationship of chemokine or chemokine receptor polymorphisms and human transplant rejection have been published; however, common genetic variants of CCR5, CCR2, and CX3CR1 have been described (12–18). These include CCR5-Δ32, CCR5-59029-A/G, CCR2-V64I, CX3CR1-V249I, and CX3CR1-T280M; all except CCR2-64I are known to affect chemokine receptor function and/or expression in primary cells. Here we analyze the association of these variants with outcome in a cohort of patients who had undergone renal transplantation.

Materials and Methods

Study Participants

We carried out a cross-sectional study on 163 renal transplant recipients who were transplanted and followed for a median of 35 mo at the Brigham and Women's Hospital outpatient clinic. Immunosuppression consisted of cyclosporine A (CsA), steroids, and mycophenolate mofetil. Humanized anti-interleukin-2 (IL-2) receptor antibody (Daclizumab; Hoffman LaRoche, Nutley, NJ) was used in 31 patients as an induction therapy to avoid using cyclosporine unless acute rejection occurred. The following information was collected for each patient: age, gender, and race of donor and recipient, type of donor

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(cadaver *versus* living), cold ischemia time, time to initial graft function, number of HLA mismatches, panel reactive antibodies, number of rejection episodes, immunosuppression therapy, and serum creatinine at discharge, 6 mo, and annually thereafter. Acute rejection was defined either as histologically proven acute rejection ($n = 58$) or an acute rise in the serum creatinine of more than 20% that responded to antirejection therapy in those patients in whom biopsy was contraindicated ($n = 5$). Acute rejection was initially treated with intravenous steroids. Steroid-resistant rejection was treated with OKT3 monoclonal antibody.

DNA Extraction

DNA was extracted by suspending the buffy coat from 5 ml of blood in 4.5 ml of red cell lysis buffer (155 mM ammonium chloride; 10 mM potassium carbonate). The pellet was resuspended by using a solution of 3 ml of white cell lysis buffer (50 μ l of 10% SDS; 50 μ l of proteinase K). Saturated sodium acetate solution was added, and the protein precipitate was spun for 15 min. The supernatant was transferred, and DNA was precipitated by using an equal amount of cold isopropyl alcohol.

Determination of CCR5- Δ 32 Genotype

CCR5- Δ 32 genotype was determined by sizing PCR amplicons that include the entire region of the deletion, a modification of methods we have used previously (13). PCR was conducted in a 15- μ l reaction containing 50 ng of genomic DNA, 5 pmol of each primer, 175 μ M deoxynucleotide triphosphates, 1.5 mM magnesium chloride, 1 \times PCR buffer, and 0.5 U of Platinum *Taq* polymerase (Invitrogen, La Jolla, CA). Thermocycling procedure (PTC 100; MJ Research, Watertown, MA) consisted of initial denaturation at 94°C for 4 min followed by 35 cycles of 94°C for 30 s, 52°C for 45 s, and 72°C for 1 min, and final extension at 72°C for 7 min. Amplicons were visualized by ultraviolet transillumination in 2% agarose gel containing ethidium bromide. The sense primer was 5'-TGTTT-GCGTCTCTCCCAG-3', and antisense was 5'-CACAGCCCTGTGC-CTCTT-3', which result in a 233-bp product for the wild-type amplicon and 201-bp for the deletion product.

Determination of CCR5-59029 Genotype

CCR5-59029 genomic variants were detected by using PCR followed by restriction enzyme fragment analysis (PCR-RFLP), a slight modification of our previously published procedure (14). The sense primer, 5'-CCCGTGAGCCCATAGTTAAACTC-3', and antisense primer, 5'-TCACAG/G'G'CTTTTCAACAG'TAAG'G'-3', pair were used with PCR conditions identical to those for CCR5- Δ 32 except for an annealing temperature of 65°C. The reaction yields a 268-bp amplicon. A total of 10 μ l of PCR product was digested with 10 units of *Bsp*1286I (New England BioLabs, Beverly, MA) per the manufacturer's recommendations. The presence of the G nucleotide at position 59029 of the CCR5 gene creates a recognition site for the *Bsp*1286I enzyme. Cut amplicons from homozygotes for 52909-G appear as a single ~130-bp band on agarose gel electrophoresis, homozygotes for 59029-A appear as a 258-bp band, and heterozygotes have both bands.

Determination of CCR2-V64I Genotype

Genotyping was performed as originally described by Smith *et al.* (15) with some minor modifications. PCR was performed as above except for the use of an annealing temperature of 65°C. The sense primer was 5'-TTGGTTTTGTGGGCAACATGATGG-3', and the antisense primer was 5'-CATTGCATTCCCAAAGACCCACTC-3'. Amplification results in a 173-bp product, 5 μ l of which was then digested in a 25- μ l reaction for 3 h with 4 units of *Bsa*BI (New England BioLabs, Beverly, MA) per the manufacturer's recommendations. An A at nucleotide position 190 encodes isoleucine at amino acid position 64 and yields restriction frag-

ments of 149 and 24 bp after *Bsa*BI digestion. In contrast, the 173-bp amplicon remains uncut if a G encoding a valine is present.

Determination of CX3CR1-V249I and CX3CR1-T280M Genotypes

Genotyping was performed exactly as described previously (19).

Statistical Analyses

Patients were genotyped and followed for renal function, acute renal allograft rejection, and number of acute rejection episodes. Serum creatinine at 6 mo and 3 yr were used as indicators of renal function. Patient genotypes were used as categorical independent variables in analyzing continuous outcome variables (serum creatinine at 3 yr), the binary variable (presence or absence of a rejection episode), and the ordinal variable (number of acute rejection episodes). Nominal logistic fit was used to assess the association of chemokine receptor genotypes with the incidence of acute rejection. Number of HLA A, B, and DR mismatches, race and age of the recipients, panel reactive antibodies, cold ischemia time, immunosuppressive regimen (with or without calcineurin inhibitors), history of previous transplant (yes/no), type of transplant (cadaveric *versus* living), and delayed graft function (yes/no) were analyzed in this multivariate analysis. ANOVA was used when the effect of two independent variables (factors) on a dependent numerical variable was examined, while adjusting for a confounding covariate. All data are expressed as mean \pm SD, and $P < 0.05$ was considered significant. No correction was made for multiple comparisons. Data were analyzed using the statistical package JMP (SAS Institute, Cary, NC). Contingency analysis was performed by using a two-sided Fisher's exact test and Wolff's approximation to determine the 95% confidence interval (CI) using the program InStat3 (GraphPad Software, San Diego, CA).

Results

Recipient and donor demographics are summarized in Table 1. Transplant characteristics for the population are shown in Table 2. Thirty-four percent of the allografts were from living related donors. Seventy-five percent of the recipients had good early function, and posttransplant dialysis was not required. Table 3 summarizes the observed genotype frequencies of CCR5-59029, CCR5- Δ 32, CX3CR1-V249I, CX3CR1-T280M, and CCR2-V64I, which were consistent with those previously reported (12–17). Observed frequencies for each genotype were not significantly different from the frequencies expected under Hardy-Weinberg equilibrium conditions. A Pearson χ^2 contingency analysis of CCR5-59029 by CCR5- Δ 32 and CCR2-64I revealed that both the CCR2-64I allele and CCR5- Δ 32 are always associated with the A allele of CCR5-59029 but the CCR2-64I allele and CCR5- Δ 32 never occur on the same haplotype as previously reported (14,15,20).

Table 1. Recipient and donor demographics

Parameter	Recipients	Donors
Men	52%	51%
Age (yr \pm SE)	48 \pm 11.9	39 \pm 13.3
African American, n (%)	23 (14%)	11 (7%)
White, n (%)	125 (77%)	133 (80%)

Table 2. Transplant data

Parameter	%
Calcineurin inhibitors	81
Delayed graft function	25
Number of rejection episodes	
0	61
1	30
>1	9
Prior transplant	8.4
0 HLA mismatches	13.5
Average HLA mismatch	2.1 ± 0.18
Living donors	34.3

Table 3. Genotype frequencies of CCR5, CCR2, and CX3CR1 variants

Genotype	Frequency	Expected (n) ^a	Observed (n) ^b
CCR5-Δ32			
+/+	0.90	147.4	147
+/ Δ 32	0.10	15.2	16
Δ 32/ Δ 32	0.00	0.4	0
CCR5-59029			
G/G	0.23	39.3	37
G/A	0.53	81.5	86
A/A	0.24	42.3	40
CCR2-V64I			
+/+	0.80	130.8	131
+/64I	0.19	30.5	30
64I/64I	0.01	1.8	2
CX3CR1-V249I			
+/+	0.50	84.0	81
+/249I	0.44	66.0	72
249I/249I	0.06	13.0	10
CX3CR1-T280M			
+/+	0.66	108.5	107
+/280M	0.32	49.0	52
280M/280M	0.02	5.5	4

^a Based on Hardy-Weinberg Equilibrium proportions given observed numbers of individuals with a particular genotype.

^b Difference between observed and expected numbers; $P > 0.60$ for all genotypes.

No linkage disequilibrium was found between the CX3CR1 polymorphisms and others reported here.

CCR2 Genotypes and Acute Rejection

Table 4 shows the relationship of the patients' CCR2-V64I genotypes with the incidence of renal allograft rejection. The percentage of recipients who had a rejection episode was more than two-fold lower in individuals possessing a CCR2-64I allele *versus* those who lacked this allele (19% for +/64I or 64I/64I *versus* 44% for +/+; odds ratio [OR], 0.30; 95% CI, 0.12 to 0.78; $P = 0.014$). Table 5 shows that there was also a

correlation between the CCR2-V64I genotypes and the number of acute rejection episodes ($P = 0.03$).

CCR5 Genotypes and Incidence of Acute Rejection

Table 4 shows the correlation between the CCR5-59029 genotypes and the incidence of acute rejection. An almost two-fold lower percentage of recipients homozygous for the 59029-A allele *versus* those possessing a 59029-G allele experienced at least one episode of rejection (23% for 59029-A/A *versus* 44% for 59029-A/G or 59029-G/G; OR, 0.37; 95% CI, 0.16 to 0.85; $P = 0.016$). Recipients with a CCR5-59029-G allele also had a higher number of rejection episodes (Table 5; $P = 0.04$). There was no difference in the incidence of rejection among recipients stratified by the presence or absence of the CCR5-Δ32 allele or by the CX3CR1-V249I and CX3CR1-T280M genotypes.

Multivariate Analyses

In multivariate analysis, CCR2-V64I (OR, 0.20; 95% CI, 0.04 to 0.80; $P = 0.032$) and 59029-A (OR, 0.26; 95% CI, 0.07 to 0.82; $P = 0.027$) remained significantly associated with the incidence of acute rejection after known risk factors were entered as covariates. These included the number of HLA mismatches, race and age of the recipients, panel reactive antibodies, cold ischemia time, immunosuppressive regimen, history of previous transplant, type of transplant, and delayed graft function. Other genetic variants, such as CCR5-Δ32, CX3CR1-V249I, and CX3CR1-T280M, continued to show no significant association after multivariate adjustment ($P = 0.98, 0.76, \text{ and } 0.56$, respectively).

Association with Renal Function Posttransplant

We were unable to demonstrate an association of any of the tested genotypes with renal function 3 yr posttransplant. Specifically, creatine clearance (CrCl) as estimated by the Cockcroft formula was nearly identical for the CCR2 and CCR5 groups (CCR2 +/+ CrCl = 58.65 ± 2.12 and CCR2 +/64I and 64I/64I CrCl = 57.2 ± 2.16 [$P = 0.40$]; CCR5 59029-A/A CrCl = 59.88 ± 2.30 and 59029-A/G and G/G CrCl = 57.23 ± 2.26 [$P = 0.35$]). This may be due to the size of the study, the length of follow up, or the type of care the patients received upon the diagnosis of acute rejection.

Discussion

Our data demonstrate the first association between human chemokine receptor polymorphisms and acute renal allograft rejection. The proportion of patients with acute rejection as well as the number of rejection episodes per patient was significantly lower in patients possessing the CCR2-64I allele and in those homozygous for the CCR5 59029-A promoter allele. These differences persisted even after correction for other risk factors. These two genotypes are in complete linkage disequilibrium (*i.e.*, the CCR2-64I polymorphism only occurs on the 59029-A allele); therefore, it is difficult to tell whether the association is due to changes in the function or expression of CCR2, CCR5, or both (14,15,20).

Association of the CCR5-59029-A allele with lower risk of acute rejection is counterintuitive. This allele has been shown to increase *in vitro* promoter activity, and 59029-A/A homozygotes have higher CD4⁺ T cell CCR5 cell surface expression (14,21).

Table 4. Incidence of acute rejection (AR) in various genotypic groups

Genotypes	No AR	AR	Odds Ratio	95% Confidence Interval	P
CCR5-Δ32					
+/+	91	56	1.3	0.45 to 3.6	0.79
+/ Δ 32 or Δ 32/ Δ 32	9	7			
CCR5-59029					
G/G or G/A	69	54	0.37	0.16 to 0.85	0.016
A/A	31	9			
CCR2-V64I					
+/+	74	57	0.30	0.12 to 0.78	0.014
+/ Δ 64I or Δ 64I/ Δ 64I	26	6			
CX3CR1-V249I					
+/+	51	30	1.1	0.61 to 2.2	0.75
+/ Δ 249I or Δ 249I/ Δ 249I	49	33			
CX3CR1-T280M					
+/+	66	41	1.0	0.54 to 2.0	1.0
+/ Δ 280M or Δ 280M/ Δ 280M	34	22			

Table 5. CCR5 and CCR2 genotypes and number of acute renal allograft rejection episodes

Gene	Polymorphism	Genotypes	Number of Rejections			P
			0	1	>1	
CCR5	59029-A/G	A/A	31	8	1	0.04
		A/G or G/G	69	40	14	
CCR2	V64I	+/+	74	44	13	0.03
		+/ Δ 64I or Δ 64I/ Δ 64I	26	4	2	

Met-RANTES treatment (which lowers CCR5 levels) is associated with greater length of renal allograft survival in MHC-incompatible rat models (10). Moreover, CCR5- Δ 32 heterozygosity, which causes less cell surface expression of CCR5 (22), had no significant association with incidence of rejection in this study. Increased CCR5 levels may potentially modulate the antiallograft immune response by altering the cytokine network.

The mechanism of action of CCR2-64I and its possible effects on renal transplant rejection also remain to be fully elucidated. There is no published evidence that the CCR2-64I polymorphism alters CCR2 expression or function on leukocytes; however, the allele has been associated with delayed progression to AIDS in HIV-positive seroconverters (15). Both CCR2 and the CCR2 ligand, MCP-1, have been shown to be markedly upregulated in renal transplant rejection (7–9). Also, MCP-1 is an important chemoattractant for monocytes, and it is possible that the polymorphism may affect the migration of monocytes into the rejecting graft. In fact, Grandaliano *et al.* (9) have shown a correlation between MCP-1 levels and monocytes in rejecting human kidney allografts and that normalization of urinary excretion of MCP-1 correlated with a positive response to antirejection treatment. CCR2 knockout mice have been shown to have impaired monocyte recruitment and decreased T cell proliferation, and they produce less interferon- γ in response to foreign antigens, leading to less inflammation in mouse experimental autoimmune encephalomyelitis (23,24). Finally, in a fully mismatched MHC murine

cardiac transplant model, CCR2 knockout mice show a doubling of allograft survival (to approximately 14 d) (5). Thus, our association with CCR2-64I may reflect a reduction in the function of CCR2 in the antiallograft immune response, and linkage disequilibrium may be responsible for the apparent association with CCR5 59029-A.

Because of the small size of our cohort and its retrospective nature, this study should be considered exploratory. Future studies will be needed to confirm these chemokine receptor polymorphism associations with transplant rejection incidence and to further clarify their mechanism of action. Precise delineation of how the chemokine system functions in renal transplantation rejection may point to new therapeutic targets and prognostic markers.

While this article was in review, Fischereder *et al.* (25) reported that homozygotes for CCR5- Δ 32 had significantly longer renal transplant survival times than CCR5 heterozygotes and CCR5 +/+ individuals. Although, we also genotyped this polymorphism, our study cannot address this finding because this genotype is relatively rare (approximately 1% of whites) and because we did not observe this genotype in our cohort.

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See related editorial, “Chemokines and Transplant Immunobiology,” on pages 821–824.