Serial Changes in the Expression of CXCR3 and CCR5 on Peripheral Blood Lymphocytes Following Human Renal Transplantation

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Objectives: **In animal models of transplantation, chemokine receptors have been shown to direct the infiltration of T cells in immune responses and inflammation and to be critical in cellular recruitment. Although the chemokine receptors CXCR3 and CCR5 and their ligands have been found during acute rejection in transplanted human kidneys, the kinetics of expression on peripheral blood lymphocytes is unknown.**

Materials and Methods: **Using a whole-blood red-cell lysis fluorescence-activated cell sorter, serial expressions of CXCR3 and CCR5 on T-cell subsets were analyzed in 19 human renal transplant recipients following transplant.**

Results: **In patients developing allograft rejection (n=6), increased expression of CXCR3 occurred on the surface of CD4+ T cells by the third day after transplant. In patients remaining rejection free (n=13), decreased expression was seen. In patients experiencing allograft rejection and in those remaining rejection free, levels of CXCR3 on CD8+ T cells and CCR5 on CD4+ and CD8+ cells remained stable throughout the study.**

Conclusions: **During allograft rejection, expression of CXCR3, but not CCR5, increases on peripheral CD4+ T cells prior to clinical evidence of allograft rejection and remains elevated for more than 2 weeks following transplantation. This may represent a specific molecular target for identifying and preventing allograft rejection.**

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Following kidney transplantation, the occurrence of acute allograft rejection is a major predictor of poor long-term graft outcome (1, 2). A key factor in the development of allograft rejection is interstitial and tubular infiltration of mononuclear cells (3). These infiltrates include T cells of an activated (CD25+) and memory (CD45RO+) phenotype (4), which are involved in direct cell-mediated immune inflammation. Major control of the selective recruitment of these circulating T-cell subsets to tissue sites is accomplished through ligation of cell-surface–expressed chemokine receptors by inflammatory chemokines produced by resident and infiltrating cells. Studies on several chemokine and chemokine receptors in human renal transplantation have shown increased expression in allograft-rejection and organ-transplant models in knockout animals using antibodies to block chemokines and receptors. Key roles have been indicated for the CXCR3 and CCR5 receptors and their chemokine ligands.

During human inflammatory conditions, these receptors are of particular importance in recruiting activated and memory T cells to sites of inflammation (5, 6). For CXCR3, virtually all T cells at the sites of nonrenal human inflammation with prominent mononuclear cell infiltrates are receptor positive, with many of these cells also coexpressing CCR5. CXCR3 is expressed by varying proportions of, and CCR5 by a minority of, peripheral blood T cells, but these cells will only mediate adhesion under conditions of flow and will only migrate toward targeting chemokines following cell activation (7- 9).

Both receptors have been identified in human allograft rejection. T cells infiltrating tubulointerstitial sites in rejecting renal allografts have been shown to express CXCR3 and CCR5 (10-12); in rejected human cardiac allografts, CXCR3 is expressed by infiltrating T cells (13). This has been confirmed in animal models of transplantation in which both CXCR3 and CCR5

show increased expression during allograft rejection (14-16). The most convincing evidence of the central role of these receptors is seen in studies using knockout animals. Transplanting of wild-type hearts to receptor knockout recipients results in 7-fold and 3-fold increases in survival of CXCR3-/- and CCR5-/- mice, respectively (17, 18). This increased survival rate was consistent with the histology of the graft, which, when compared with controls, showed greatly decreased cellular infiltration and damage.

Studies also have shown that the ligating chemokines for these receptors (particularly CXCL10/IP-10 and CXCL9/Mig for CXCR3 and RANTES/CCL5 and MIP-1α/CCL3 for CCR5) are increased in transplanted kidneys during acute rejection (19-22). These studies indicate a key role for the chemokine receptors CXCR3 and CCR5 in allograft rejection.

While most studies have focused on events occurring within the graft at the time of allograft rejection, little data exist regarding the events in the peripheral blood of allograft recipients following transplantation. The aim of this study was to identify the serial changes in the expression of chemokine receptors CXCR3 and CCR5 on the surfaces of circulating T-cell subsets following transplantation and prior to the clinical development of allograft rejection.

Subjects and Methods

Venous blood was collected for fluorescence-activated cell sorter (FACS) analysis at days 0, 3, 7, 14, and 21 in Li-EDTA tubes from 19 patients (10 men, 9 women; mean age, 41 years; age range, 19-61 years) receiving a first renal transplant from deceased donors. The immunosuppressive regimen for all patients consisted of cyclosporine, azathioprine, and prednisolone with no induction antibodies. Target levels and dosages were identical for all patients. Only on the histologic diagnosis of rejection, which was confirmed on the day of biopsy, were pulsed steroids given. Written, informed consent was obtained from all participants, and the local ethics committee of the University of Birmingham approved the study protocol (in accordance with the ethical guidelines of the 1975 Helsinki Declaration).

FACS analysis was performed using a FACScalibur flow cytometer (Becton Dickinson, Cowley, UK) calibrated with beads labeled with the 3 fluorochromes used. All antibodies were added to 50 μL of whole blood and incubated for 15 minutes at room temperature. We used mouse monoclonal antibodies directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or peridinin chlorophyll protein (PerCP) as follows: CD4-FITC, CD8-FITC, CXCR3-PE (R&D systems, Abingdon, UK at 2.5 μg/mL), CD3-PerCP, CD4-FITC/CD8-PE SimulTEST (at 1:50), CD3-CD4-CD45 Tritest (at 1:50), and CD3-CD8-CD45 Tritest (at 1:50). Labeled isotype control antibodies were used for all antibodies at equivalent concentrations. Red blood cells were then removed through incubation with red-cell lysis solution (FACSLysis) for 10 minutes, centrifuged at 1000 rpm for 5 minutes, and washed with phosphate buffered saline containing 0.1% NaN_3. Subsequent gating and analyses were performed using WinMDI 8.0 software (The Scripps Institute, Flow Cytometry Core Facility, San Diego, CA). Unless otherwise stated, all antibodies and FACS reagents were from Becton Dickinson Biosciences (Franklin Lakes, NJ), and all antibodies were used at concentrations of 1 μg/mL (unless otherwise stated).

Results

Flow cytometry was used to analyze the expression of CXCR3 on T cells in whole blood. Acute rejection was confirmed by biopsy, and all episodes were classified as tubulointerstitial (Banff type 1A, n=2; 1B, n=4). No patients had vascular (Banff type 2) rejection. The decision to biopsy was based on a 20% increase in the level of serum creatinine or a daily rise of 10% on sequential days.

Samples were analyzed using scatter characteristics of the FACS plots and CD3 to allow gating on the lymphocyte population. The percentage of T cells expressing CXCR3 and CCR5 is consistent with published data (5, 7, 11). When analyzed by percentage of cell expression (ANOVA) at all time points, no differences were found regarding receptor expression between patients who remained rejection free (n=6) and those with rejection (n=13).

Geometric mean fluorescent intensity was used to assess the density of surface receptor expression at sequential time points of CD4+ and CD8+ T-cell populations (Table 1). These were standardized and are expressed as a ratio by comparing levels for each patient, at each time point, against levels from day zero (Figures 1a-d). The means of the standardized values were compared for allograft rejection+ and allograft rejection- patients for both CD4+ and CD8+ subsets. In allograft rejection+ patients, mean cell surface expression of CXCR3 was significantly increased on CD4+, but not on CD8+, T cells by 3 days

Table 1. Mean cellular fluorescence (geoMFI ± SEM) of CXCR3 and CCR5 on peripheral circulating lymphocytes. Using geometric mean cellular expression (geoMFI ± SEM) of receptor expression, both CXCR3 and CCR5 were studied on CD4+ and CD8+ T cells. No significant differences were seen in the pretransplant level in either AR+ (n=6) or AR- (n=13) patients. However, by day 3, both CD4+ and CD8+ cells showed that expression of CXCR3 had increased above baseline in those patients with subsequent AR. In the CD4+ cell subset, levels remained above baseline until day 14, at which point they decreased to below baseline by day 21. A similar but less pronounced trend occurs in CD8+ cells. In AR- patients, levels dropped following the transplant and remain low. For CCR5, geoMFI on CD4+ and CD8+ cells show minimal changes in expression prior to transplant and throughout the study in AR+ and AR- patients.

	D0 CXCR3	D3 CXCR3	D7 CXCR3	D14 CXCR3	D21 CXCR3
	CD4				
AR+	182.4± 37.7	294.9 ± 47.8	223.4 ± 37.1	187.7± 44.3	85.4 ± 20.1
AR-	256.0± 41.2	187.6 ± 28.3	193.2 ± 26.3	186.4± 31.3	170.6 ± 22.2
	CD8				
AR+	163.4 ± 23.1	209.2± 45.9	168.0±26.8	194.0 ± 41.5	129.2 ± 34.1
AR-	179.4 ± 31.0	138.6 ± 22.3	145.3 ± 23.1	140.0 ± 22.5	145.7± 16.3
	D0 CCR5	**D3 CCR5**	**D7 CCR5**	**D14 CCR5**	**D21 CCR5**
	CD4				
AR+	55.2 ± 17.2	56.4 ± 8.5	55.8 ± 30.4	61.8 ± 15.8	24.6 ± 16.5
AR-	67.4 ± 8.2	72.6 ± 14.3	68.3 ± 13.9	69.6 ± 17.4	66.2 ± 22.3
	CD8				
AR+	57.4 ± 8.7	46.4 ± 8.5	52.4 ± 18.2	51.0 ± 6.6	46.0 ± 7.3
AR-	75.3 ± 9.5	67.3 ± 9.7	75.5 ± 9.8	77.8 ± 13.3	79.8 ± 7.1

Abbreviations: GeoMFI, geometric mean fluorescent intensity; SEM, standard error of the mean; AR, allograft rejection; D, day

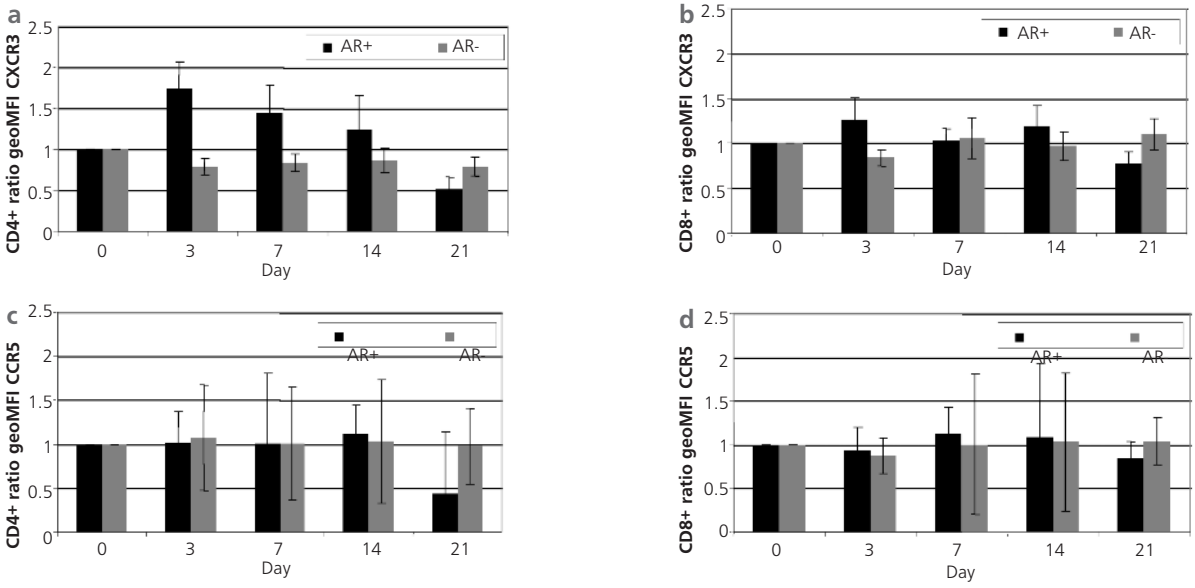

Figure 1. Mean ratio of geoMFI for each time point compared with day 0 levels for AR- and AR+ patients. To provide internal control, analysis of CXCR3 and CCR5 was performed using the pretransplant level as a baseline and expressing subsequent time points as a ratio compared with this level. (a) CD4+ cells demonstrate early expression of CXCR3, which is maintained above baseline until after day 14. In AR+ patients (n=6), the median time to treatment with high-dose steroids was 13 days, and the decrease in expression may be attributed to this. (b) CD8+ cells do not demonstrate significant changes in expression when normalized to baseline expression in either AR+ or AR- patients. CCR5 expression on (c) CD4+ and (d) CD8+ cells remains similar to baseline throughout. In all AR+ patients, the level of expression dropped following day 14 and might have been the result of high-dose pulsed steroid treatment.
Abbreviations: GeoMFI, geometric mean fluorescent intensity; SEM, standard error of the mean; AR, allograft rejection

after transplant and remained elevated at day 14 (ANOVA, $P < .005$). At day 21, levels had decreased to below their pretransplant values. The median time to the diagnosis of rejection and treatment with pulsed high-dose steroids (methylprednisolone 200 mg × 3 days) was 15 days (range, 8-19 days).

In patients who remained rejection free, levels of CXCR3 and CCR5 were decreased from baseline at day 3 and remained below pretransplant levels for the duration of study.

Discussion

Our results demonstrate that in renal transplant recipients, expression of CXCR3, but not CCR5, in peripheral blood CD4+ T cells is increased in the days

following renal transplant prior to the development of allograft rejection. Previous studies have shown that T cells expressing this receptor are present within the graft on infiltrating T cells of activated and memory phenotypes and local to areas of CXCL10/IP-10 expression. This supports a role for CXCR3/CXCL10 in recruiting alloactivated T cells into engrafted tissue and the consequent development of allograft rejection.

Infiltration of T cells to tubular and interstitial sites within the renal cortex depends on the ligation of T-cell-expressed chemokine receptors by inflammatory chemokines. Although previous studies have shown up-regulation of several chemokines and their receptors at tissue sites during allograft rejection (10-14), the current study analyzed serial changes in the expression of CXCR3 and CCR5 in peripheral blood following renal transplant. Dalton and associates recently analyzed serial chemokine and chemokine receptor gene expression following renal transplant, although they failed to demonstrate significant expression of either CXCR3 or CCR5; this may reflect differences between receptor production and surface expression (20). The potential relevance of increased CXCR3 expression is demonstrated by recent data from a mismatched murine cardiac transplant model that indicates that CXCR3/CXCL10 is the dominant chemokine/ligand pairing in the development of allograft rejection (16). While CXCR3 may be central to the initial recruiting of alloactivated cells, once present at tissue sites, other chemokines/chemokine receptor pairs, such as CCR5 and its ligands, may be responsible for tissue migration and retention.

Although to date there have been no detailed analyses of the kinetics of chemokine receptor expression, previous studies on circulating T cells for expression markers for the development of allograft rejection show variable associations (17). Because their presence on activated T cells is a major determinant of the ability of T cells to migrate to inflammatory sites, we were interested in CXCR3 and CCR5 expression (5, 7, 10). We hypothesized that increased expression of CXCR3 on circulating T-cell subsets in human renal transplant recipients may follow priming of the cells with graft-specific antigens by antigen-presenting cells at secondary lymphoid sites in the days following transplant. In a sequential analysis, we show increased expression of CXCR3 on CD4+ T cells by day 3 in the peripheral blood of patients who developed allograft rejection following transplantation, which was then sustained until day 14. By day 21, expressions of CXCR3 and CCR5 had fallen to below baseline, which might reflect the effect of the high-dose steroids used to treat episodes of allograft rejection (which are known to down-regulate chemokine receptor expression on inflammatory cells) (20). The median time of treatment of acute rejection for these patients was day 15. As CD4+ T cells are central in promoting infiltration-dependent inflammatory injury in engrafted tissue, increased levels of CXCR3 may lower the threshold for recruiting this T-cell subset and may be an important determinant of the development of allograft rejection. We failed to show a statistically significant increase in CXCR3 expression on the surface of circulating CD8+ T cells; this may reflect the relative balance of alloproliferation at secondary lymphoid sites between CD4+ and CD8+ cells.

Previous studies have examined intragraft CXCR3 expression and ligating chemokines in human lung and heart allografts. Agostini and colleagues recently found infiltrating T cells expressing CXCR3 within rejecting human lung allografts; these T cells were highly responsive to CXCL10/IP-10, which was present in the bronchoalveolar fluid of these patients in large quantities (13). In human renal disease, Romagnani and colleagues recently showed the presence of CXCR3 in human glomerulonephritis, principally at intraglomerular sites (21). Hancock and colleagues correlated CXCR3 expression with CD3+ T-cell infiltration and acute rejection following human cardiac transplant (17) with concurrent heavy intragraft expression of CXCL10/IP-10 mRNA. In human studies, Hu and colleagues confirmed increased expression of CXCL10/IP-10 in the urine of patients with acute allograft dysfunction; however, this was not specific to allograft rejection (22).

Compared with CXCR3, CCR5 appears to play less of a role in the initial recruitment of T cells to tissue sites in allograft rejection. CCR5 may be involved in posttrafficking events within the graft. The importance of CCR5 is demonstrated both in knockout animals (where survival is significantly prolonged when compared with wild-type recipients) and in epidemiologic studies of CCR5 allotypes. In approximately 1% of the population, a base pair deletion, the delta 32 deletion, occurs and results in a lack of CCR5 expression. Although allograft rejection occurred in over 40% of patients with this deletion, they demonstrated significantly better long-term outcomes following renal transplantation when compared with patients expressing functional CCR5 (23). The interactions of these chemokine ligands and receptor pairings also may play a role in the initiation and propagation of late and recurrent rejection episodes, although further studies are required to demonstrate whether other chemokine pathways are recruited.

In conclusion, we have shown that in renal transplant recipients who develop acute allograft rejection, the development of rejection is preceded by increased expression of CXCR3 on circulating CD4+ T cells. These observations are consistent with animal studies that indicate a key pathway for CXCR3 and its ligands in acute allograft rejection. These data reinforce the potential to target highly specific receptor ligand pairings for prophylaxis of allograft rejection in human transplants and may have significance with respect to the noninvasive diagnosis and pre-emptive biopsy or treatment of rejection. Further studies are required to expand upon our results.

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