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ORIGINAL ARTICLE

Role of CXCR3 in cellular but not humoral renal allograft rejection

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Summary

CXCR3, a chemokine receptor mainly expressed by T cells, is involved in animal transplant models and in human allograft rejection. CXCR3 expression was localized in formalin-fixed, paraffin-embedded renal allograft biopsies without signs of rejection (C4d-negative, Banff 0, $n = 16$), with C4d deposits as a sign of humoral rejection (C4d-positive, Banff 0, $n = 8$), with cellular rejection (C4d-negative, Banff I, $n = 7$) and with signs of both cellular and humoral rejection (C4d-positive, Banff 1, $n = 5$). Small, round infiltrating cells were CXCR3-positive. A high number of these cells was present in biopsies with cellular rejection (independent of C4d deposition). CXCR3-positive cells diffusely infiltrated the interstitium, including the tubular epithelium (tubulitis). CXCR3 scores and the area of CXCR3 staining were significantly higher in cellular rejection, when compared to biopsies without rejection, and with deposition of C4d alone. CXCR3-positive cells infiltrate renal allografts during cellular rejection, whereas C4d deposition is not associated with the recruitment of these cells.

Introduction

The recruitment of inflammatory cells into renal allografts is a hallmark of acute allograft rejection [1]. This process is mediated through the interaction of chemokines and the corresponding chemokine receptors with adhesion molecules, in a multistep process [2–6]. During recent years the role of chemokines in allograft rejection has been addressed in numerous studies *in vitro*, in animal models, and in biopsy tissue from allografts [4–8]. Detailed studies of a heterotopic, major histocompatibility complex (MHC)-mismatched, heart transplant model have been performed, with the use of genetically engineered mice. These studies illustrate a major role of the chemokine receptors CXCR3 and CCR5 during allograft rejection, as deficiency of these receptors in the recipients prolonged allograft survival [4,7,8]. CXCR3 is a receptor expressed on activated T cells (mainly of the T-helper type 1), on some B cells and natural killer cells [9]. CXCR3 binds the ligands CXCL9/MIG, CXCL10/IP-10 and CXCL11/I-TAC [9].

In the heart transplantation model the deficiency of single chemokines of the recipient usually does not protect from allograft rejection [4]. Accordingly, the CXCL10/IP-10-deficient recipients rejected the allografts similarly as the wild-type controls [7]. In contrast, when donor hearts from CXCL10/IP-10-deficient mice were transplanted into wild-type recipients, the allografts survived long term [7]. Furthermore, blockade of CXCL10/IP-10 improved allograft survival, implying a therapeutic impact [7]. In CXCR3-deficient recipients the allograft survival was significantly prolonged, and combination with subtherapeutic doses of cyclosporin A lead to acceptance of the graft [8]. These elegant studies provide clear evidence for an important role of CXCL10/IP-10 released by donor cells, and CXCR3 expressed on recipient cells

during allograft rejection. CXCL10/IP-10 mRNA has been shown to be induced in renal nephrectomy specimens, and renal biopsies with signs of acute rejection [5,10]. Furthermore, excretion of CXCR3-positive cells and of the CXCR3 ligands in urine is increased during renal allograft dysfunction, offering a tool for allograft monitoring [11,12]. Finally, CXCR3-positive cells have been shown to be involved in the process of transplant glomerulopathy [13]. However, the distribution of CXCR3-positive cells has not been described in renal biopsies with antibody-mediated rejection in comparison with cellular rejection. We therefore performed immunohistochemistry for CXCR3 in biopsies with different forms of human renal allograft rejection.

Materials and methods

Immunohistochemistry for CXCR3 and C4d was performed on archival formalin-fixed, paraffin-embedded renal biopsies using the monoclonal antihuman CXCR3 antibody 1C6 (BD Biosciences Pharmingen, Heidelberg, Germany), and a polyclonal rabbit serum against C4d (C4dpAb, Biomedica, Vienna, Austria). The biopsies included in this study have previously been described in detail [14]. Biopsies without signs of allograft rejection (Banff 0/borderline, C4d-negative, $n = 16$), were compared to biopsies with cellular rejection (Banff 1, C4d-negative, $n = 7$), with biopsies suspicious of humoral rejection (Banff 0, C4d-positive, $n = 8$) and with biopsies that demonstrated signs of cellular rejection and C4d deposits (Banff 1, C4d-positive, $n = 5$) [15]. To characterize the distribution of CXCR3-positive cells, we used a monoclonal antibody against CXCR3 (Clone:1C6, BD Biosciences Pharmingen) for immunohistochemistry as previously described [16]. Controls with an isotype-matched immunoglobulin G (IgG) were performed on selected biopsies as well as on tissue sections from tonsils and allograft nephrectomies. In brief, the 2 μm tissue sections were dewaxed, and rehydrated. Endogenous peroxidase was blocked by 3% hydrogen peroxide. Antigen retrieval was performed by autoclaving. The Avidin/Biotin Blocking Kit (Vector, Burlingame, CA, USA) was used to block endogenous biotin. The primary antibody was added for 1 h, followed by incubation with a biotinylated antimouse IgG antibody (Vector) and the ABC reagent (Vector). 3′,3′-Diaminobenzidine (DAB; Sigma, Taufkirchen, Germany) with metal enhancement (resulting in a black colour product) served as detection system. Slides were counterstained with methyl green, dehydrated and mounted.

Specimens were scored without knowledge of the diagnosis for CXCR3-positive cells semiquantitatively from 0 to 3+ (0: only scattered positive cells, 1: mild, focal infiltrates, 2: moderate infiltrates with at times several positive cells between tubuli, 3: strong, diffuse infiltrates at times with confluent cell aggregates). Additionally, to the semiquantitative scoring of each biopsy the slides were evaluated by morphometrical analysis. About 15 consecutive high power fields (at a magnification of ×20) each measuring 0.152 mm^2 were analysed for each biopsy. The area of positive colour product was expressed as percentage of the area of the high power field. The measurement was performed using a digital camera and image analysis software (Leica Qwin, Cambridge, England).

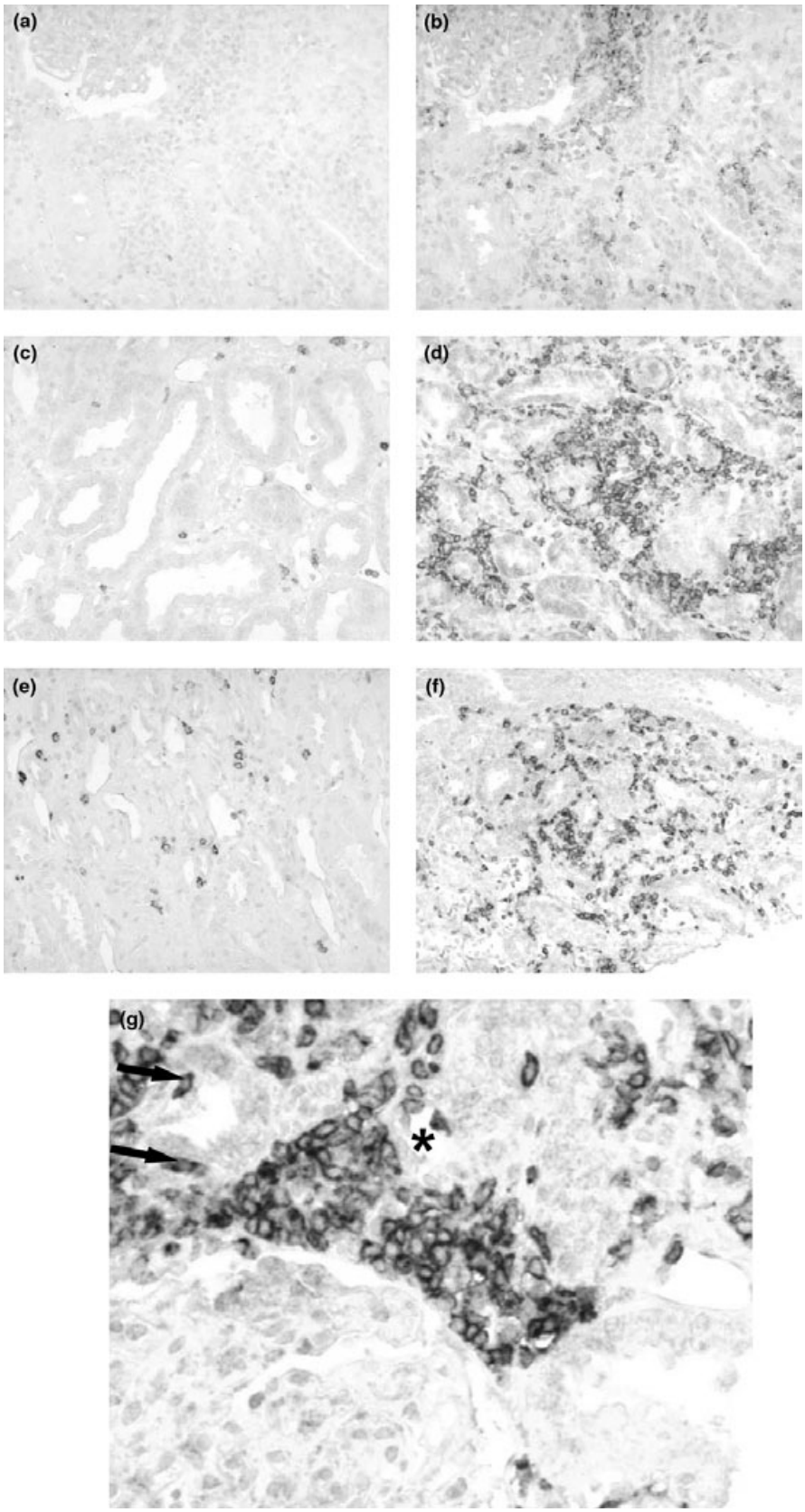
The statistical analysis was performed with the INSTAT® program (Version 3.05 for Windows, Intuitive Software for Science, San Diego, CA, USA). The nonparametric Kruskal–Wallis test and the Dunn's multiple comparison test were used for the comparison of the mean values. A $P < 0.05$ was considered to be statistically significant.

Results

CXCR3 expression could be detected on a population of small round infiltrating inflammatory cells, but not on intrinsic renal cells in renal biopsies (Fig. 1b). The distribution pattern, and morphology in renal biopsies, human tonsils and allograft nephrectomies was consistent with infiltrating lymphocytes (not shown). The isotype controls did not demonstrate a positive signal (Fig. 1a).

CXCR3-positive cells were mainly found in peritubular capillaries, in small focal interstitial infiltrates, and rarely in glomerular capillaries in biopsies without signs of rejection (Fig. 1c). A similar pattern was detected in renal biopsies from patients with C4d deposition in peritubular capillaries (Fig. 1e). In contrast to biopsies from these two groups, a prominent influx of CXCR3-positive cells was found in biopsies with acute cellular rejection (Fig. 1d,f,g). Acute cellular rejection lead to a diffuse infiltration of the tubulointerstitium with CXCR3-positive cells (Fig. 1d). These cells at times formed dense aggregates between tubuli and around glomeruli (Fig. 1g). CXCR3-positive cells infiltrated the tubular epithelium (tubulitis, Fig. 1g). Furthermore, these cells could be seen to be attached to the endothelium of peritubular capillaries. There was no apparent difference between the distribution of CXCR3-positive cells in biopsies with 'pure' cellular rejection, and with a combination of cellular rejection and C4d deposits (Fig. 1d,f).

Comparison of the semiquantitative scores of CXCR3-positive infiltrates in the four groups demonstrated that only cellular rejection lead to a significant increase of the scores, consistent with the morphological description. Biopsies with C4d deposits showed similar scores as biopsies without rejection. Furthermore, the mean scores from biopsies with cellular rejection and C4d deposits did not


(a)
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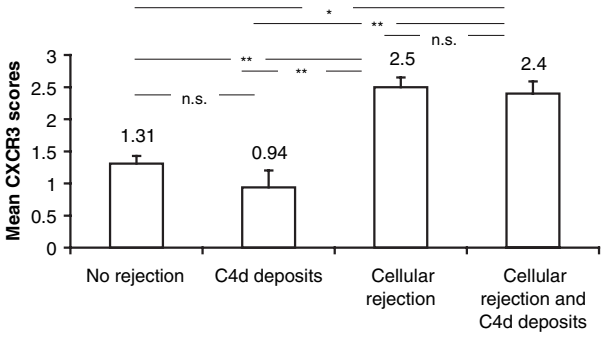

Figure 2 Mean CXCR3 scores in the four diagnostic groups (error bars, SEM; NS, not significant; $*P < 0.05$, $**P < 0.01$).

differ significantly from the scores of biopsies with cellular rejection alone (Fig. 2). Using morphometrical analysis of the area of positive staining there was no difference between biopsies without rejection and biopsies with C4d deposits ($1.0 \pm 0.22\%$ vs. $0.89 \pm 0.19\%$). Furthermore, no significant difference was found in cases with cellular rejection, between biopsies with and without C4d deposits (4.0 ± 1.15 vs. $5.6 \pm 1.24\%$). In contrast, the area of positive CXCR3 staining was significantly larger in biopsies with cellular rejection when compared to biopsies with humoral rejection alone or biopsies without rejection ($P < 0.01$).

Discussion

Delayed type hypersensitivity is an important effector pathway of the T-cell response during cellular rejection of vascularized allografts (mediated through CD4+ Th1 cells [17]). The chemokine receptor CXCR3 is predominantly expressed on Th1 cells. Data on the role of CXCR3 in human renal allograft rejection are still limited. A strong expression of CXCR3 by infiltrating cells was found in biopsies from patients with cellular rejection [11,18]. Consistent with these results the current study demonstrates a significant increase in CXCR3-positive lymphocytes during cellular allograft rejection (according to the Banff classification). CXCR3 was expressed by a population of small round infiltrating cells, consistent with lymphocytes. These cells accumulate in the tubulointerstitium, between tubuli (at times forming dense aggregates) and are involved in tubulitis (a hallmark of acute cellular rejection). In this series, only two of 23 biopsies without cellular rejection demonstrated a positive CXCR3 staining above 1.5% of the biopsy. Only two of 12 biopsies with cellular rejection demonstrated a positive staining below 1.5%. For the diagnosis of cellular rejection this test with a cut off of 1.5% positive CXCR3 staining area would result in a sensitivity of 0.91 (0.72–0.99), a specificity of 0.83 (0.52–0.98), a positive predictive value of 0.91 (0.72–0.99) and a negative predictive value of 0.83 (0.52–0.99). Furthermore, none of the C4d-positive biopsies without signs of cellular allograft rejection demonstrated a CXCR3-positive staining area above 1.5%. Therefore, the current study implies that staining of routine formalin-fixed, paraffin-embedded allograft biopsies for CXCR3 is possible and CXCR3 should be evaluated further as a diagnostic marker. A note of caution needs to be added herein, as it is very difficult to define the sensitivity of the staining procedure (i.e. the expression of CXCR3 needed that results in a positive colour product). We might underestimate the number of CXCR3-positive cells by immunohistochemistry.

Another effector pathway contributing to renal allograft injury, i.e. antibody-mediated allograft rejection, has attracted increasing attention during recent years [15,19]. This form of allograft rejection is characterized by the deposition of the complement split product C4d in peritublar capillaries, antidonor antibodies in the recipient serum, accumulation of inflammatory cells in peritubular capillaries and a poor allograft outcome [19]. According to an addition of the Banff 97 classification the deposition of C4d should be entitled as being suspicious for humoral rejection, without documentation of antidonor antibodies in the serum of the recipient [15]. In our experience, almost 90% of the patients who demonstrate C4d deposition have detectable antidonor antibodies using fluorescence-activated cell sorter (FACS) cross-match testing [20].

In the current study, biopsies with C4d deposits demonstrated similar CXCR3 scores and CXCR3-positive staining area as normal controls. Therefore, the humoral mechanism of allograft injury was not associated with the recruitment of CXCR3-positive cells. In contrast in a previous study, it was shown that the expression of a chemokine-binding protein (the Duffy Antigen Receptor for Chemokines, DARC), was increased in biopsies with C4d

Figure 1 Immunohistochemistry performed with control immunoglobulin G (IgG; a) and the monoclonal anti-CXCR3 antibody (b–g). (a, b) Consecutive sections of a renal allograft biopsy from a patient with Banff 1 acute allograft rejection demonstrated diffuse infiltration by CXCR3-positive cells (b), but no signal was detectable on the slide incubated with the istotype control (a, original magnification ×200). (c–f) Biopsies were from patients without allograft rejection (c), with cellular allograft rejection (d), with C4d deposition in peritubular capillaries (e) and with both cellular allograft rejection and C4d deposits (f). Please note the similar pattern of scattered positive cells in biopsies without rejection and with C4d deposits (c and e). A prominent influx of CXCR3-positive infiltrating cells was present in biopsies with cellular rejection (d, f). (g) A periglomerular infiltrate consisting of CXCR3-positive cells, with infiltration of the tubular epithelium (arrows) and positive cells attached to the endothelium of a peritubular capillary (*).

deposits [14]. Endothelial DARC might be involved in transferring certain chemokines from the abluminal to the luminal side of endothelial cells, and present these chemokines on the luminal side. Interestingly, DARC does not bind the ligand CXCL10/IP-10 for CXCR3. Therefore, it was expected that increased expression of DARC is not associated with the recruitment of CXCR3-positive cells, as shown by the current study. As the main cell type accumulating in peritubular capillaries are monocytes/macrophages during acute humoral rejection, it can be hypothesized that expression of DARC might be important for the presentation of chemokines attracting macrophages as CCL2/MCP-1.

Conclusions

The data are consistent with the hypothesis of CXCR3 being a marker of a Th1 type immune responses, whereas the humoral response does not involve CXCR3 expression in the graft. The main findings of the current study are that CXCR3-positive cells are involved in cellular rejection of human renal allografts, consistent with experimental data, and complement activation on the endothelium of peritubular capillaries is not associated with the recruitment of CXCR3-positive cells. Different expression patterns of chemokines and chemokine-binding proteins in cellular and humoral rejection might be important for the differences in the inflammatory cell influx. CXCR3 might be a diagnostic tool as well as an attractive therapeutic target during acute cellular rejection.

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