

WHEN RENAL ALLOGRAFTS TURN DARC¹

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Background. The Duffy antigen-receptor for chemokines (DARC) is a chemokine-binding protein that is up-regulated on peritubular capillaries (PTC) during cellular renal allograft rejection. C4d deposition and accumulation of inflammatory cells in PTC are indicators of humoral renal allograft rejection. Because DARC is expressed at the site of C4d deposition and might be involved in inflammatory cell recruitment, the authors evaluated the expression of DARC in different forms of human renal allograft rejection.

Methods. Deposition of C4d and DARC expression were evaluated by immunohistochemistry in 42 renal transplant biopsy specimens. Biopsy specimens were subdivided according to histologic and immunohistochemical results, that is, C4d-negative biopsy specimens with (Banff 1, n=8) or without signs of cellular rejection (n=16), and C4d-positive biopsies (humoral rejection) with (Banff 1 rejection, n=7) or without cellular rejection (n=11).

Results. DARC expression was found on a small number of PTC and veins in patients without rejection. Cellular and humoral rejection led to a comparable increase in the number of DARC-positive PTC (9.7 and 8.7 vs. 2.6 vessels per high-power field [HPF], respectively). The highest numbers were found in biopsy specimens with signs of both humoral and cellular rejection (17.5 vessels per HPF).

Conclusions. This is the first study that demonstrates an induction of a chemokine-binding protein at the site of C4d deposition in humoral allograft rejection. The additive effect of humoral and cellular rejection on DARC expression might imply different pathways of DARC induction for different forms of allograft rejection.

Recruitment of inflammatory cells to specific microenvironments is mediated through a cascade of interactions between adhesion molecules, soluble mediators, and their corresponding receptors expressed on inflammatory cells and activated endothelial cells (1). In this process, chemokines

play a pivotal role in the recruitment of inflammatory cells into renal allografts (2–6). The extravasation of lymphocytes in rejecting renal allografts is thought to take place in activated peritubular capillaries (PTC) (7).

The Duffy antigen-receptor for chemokines (DARC) is a seven-transmembrane-spanning protein that, in contrast to other chemokine receptors, binds chemokines of different subgroups (8). For example the CXC chemokines IL-8/CXCL8, GRO- α /CXCL1, PF4/CXCL4, NAP-2/CXCL7, and the CC chemokines MCP-1/CCL2 and RANTES/CCL5 all bind to DARC (9, 10). An increased expression of MCP-1/CCL2, RANTES/CCL5, and IL-8/CXCL8 during allograft rejection, and accumulation of inflammatory cells expressing the corresponding receptors has been demonstrated by the authors and others (3, 11–13). DARC is currently referred to as a chemokine-binding protein and not as a chemokine receptor, because signaling on binding of chemokines has not been demonstrated (14). On red blood cells, DARC carries the Duffy blood group and might be involved in the clearance of chemokines from the circulation (8, 9). Besides red blood cells, DARC is expressed on specialized endothelial cells such as high endothelial venules in lymphoid tissue and peritubular capillaries in the kidney, where extravasation takes place (15, 16). An increased number of DARC-positive PTC has been described in human immunodeficiency virus-associated thrombotic microangiopathy, in crescentic glomerulonephritis, and in acute renal allograft rejection (17, 18). Therefore, DARC is up-regulated at the site of cell recruitment and binds chemoattractants for the most abundant inflammatory cell types involved in renal allograft rejection.

The pathogenetic role of humoral immune mechanisms in acute renal allograft rejection has become apparent during recent years (19, 20). Deposition of the complement split-product C4d in PTC is now a well-established indicator of acute humoral allograft rejection (21–24). There is evidence that positive C4d staining is associated with inferior renal allograft outcome and the occurrence of immunologic graft loss (21–25). Because deposition of C4d might indicate an activation of peritubular capillary endothelium and DARC might be involved in the accumulation of inflammatory cells at this site, the authors compared the DARC expression between humoral and cellular allograft rejection. In this study, the authors addressed the following questions: (1) Is DARC expression induced by humoral alloresponses? (2) Is there a quantitative difference of DARC-positive vessels between cellular and humoral allograft rejection? (3) What is the impact of a mixed pattern of humoral and cellular rejection on DARC expression?

MATERIALS AND METHODS

Study Population

After routine workup, 42 human renal allograft biopsy specimens were included in the study. The biopsy specimens were from 16 patients without morphologic evidence of allograft rejection (Banff 0

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or borderline, no C4d deposition in PTC), eight patients with cellular allograft rejection (Banff 1) without C4d deposition, 11 patients with C4d deposition in PTC without signs of cellular allograft rejection according to Banff criteria (humoral rejection), and seven patients with the morphologic diagnosis of both Banff 1 allograft rejection and C4d deposition in PTC.

Immunosuppressive Therapy

1. Patients without allograft rejection (n=16): Fourteen patients received basal immunosuppression consisting of cyclosporine A (CsA), mycophenolate mofetil (MMF) or azathioprine, and steroids. Four of these recipients received induction therapy with antithymocyte globulin (ATG) (n=3) or basiliximab (n=1). Two patients received rapamycin, CsA, and steroids.
2. Patients with acute cellular rejection (n=8): Recipients from this group were on CsA, MMF or rapamycin (n=2), and steroids.
3. Patients with humoral rejection (n=11): Nine patients were on CsA, MMF, and steroids. Four of these recipients received induction therapy with ATG and one with basiliximab. One patient received triple therapy with rapamycin instead of MMF and one patient with tacrolimus instead of CsA.
4. Patients with combined humoral and cellular rejection (n=7): Five patients were on CsA, MMF, and steroids. One patient received rapamycin-based and one received tacrolimus-based immunosuppression.

Immunohistochemistry

DARC expression was evaluated on formalin-fixed, paraffin-embedded material using a monoclonal mouse antibody (2C3), similar to that described previously (3). In brief, 2- μ m tissue sections were dewaxed in xylene and rehydrated in a graded series of ethanol. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide. Antigen retrieval was performed in Antigen Retrieval Solution (Vector Laboratories, Burlingame, CA) in a microwave oven. Endogenous biotin was blocked using the Avidin/Biotin Blocking Kit (Vector). Incubation with the primary antibody (2C3) was followed by incubation by an biotinylated anti-mouse immunoglobulin (Ig) G antibody (Vector) and the ABC reagent (Vector). 3'3'Diaminobenzidine (DAB; Sigma, Taufkirchen, Germany) with metal enhancement (resulting in a black color product) was used as a detection system. Slides were counterstained with methyl green, dehydrated, and mounted in Histomount (Zymed Laboratories, San Francisco, CA).

Positive vessels in the tubulointerstitium were counted in each biopsy specimen in up to 15 high-power fields ($\times 400$) by an observer blinded to the diagnosis of the biopsy specimen. The data were confirmed by a second "blinded" observer. The numbers are given as means and SEM.

As negative controls, the primary antibody was replaced by isotype-matched irrelevant mouse IgG on tissue sections from human tonsil and selected transplant biopsy specimens. A black color product was detectable on endothelial cells in biopsy specimens stained with the monoclonal anti-DARC antibody (2C3), but never in IgG isotype controls (Fig. 1A and B). By immunofluorescence, DARC staining was localized to the endothelial surface, with an accentuation at cell-cell junctions. In addition, some DARC reactivity was detected in the cytoplasm using immunofluorescence microscopy (data not shown).

For C4d staining, the authors used immunohistochemical staining of sections from formalin-fixed, paraffin-embedded allograft biopsy specimens using a rabbit polyclonal anti-C4d antibody (C4dAb; Biomedica, Austria) as previously described (24). The polyclonal antiserum was raised against a 15mer peptide corresponding to amino acids 1242 to 1256 of C4 (24). Only C4d deposition in PTC was regarded as being diagnostic for acute humoral allograft rejection, but not the deposition in glomeruli or larger vessels. Biopsy specimens with circumferential linear endothelial C4d staining in at least 25% of PTC were classified as being C4d-positive.

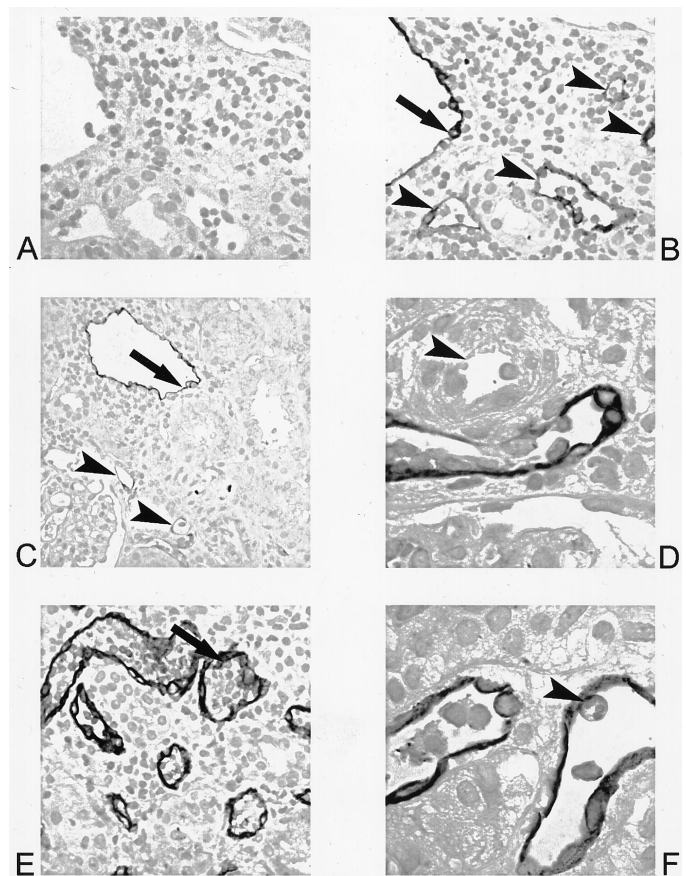


FIGURE 1. Immunohistochemistry performed with control IgG (A) and the monoclonal anti-DARC (2C3) antibody (B-F). (A and B) Consecutive sections of a renal allograft biopsy specimen from a patient with Banff 1 acute allograft rejection demonstrates a positive endothelial signal on a vein (arrow) and on four peritubular capillaries (arrowheads), but no signal was detected on the isotype control (A, magnification $\times 400$). (C) Low number of positive vessels, a larger vein (arrow), and two PTC (arrowheads) in a biopsy specimen without signs of rejection (magnification $\times 200$). (D-F) Biopsy specimen from a patient with signs of humoral and cellular allograft rejection demonstrates negative endothelium on an arteriole (D) (magnification $\times 1,000$) (arrowhead), accumulation of inflammatory cells in DARC-positive peritubular capillaries (E) (arrow) (magnification $\times 400$), and a granulocyte attached to DARC-positive endothelium (F) (arrowhead) (magnification $\times 1,000$).

Statistical Analysis

The statistical analysis was performed using the InStat program (Version 3.05 for Windows, Intuitive Software for Science, San Diego, CA). For the comparison of the means, the nonparametric Kruskal-Wallis test and the Dunn multiple comparison test were used. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

DARC Expression in Biopsy Specimens without Signs of Rejection

The control group consisted of 16 patients without deposition of C4d in the allograft biopsy specimen in PTC and without signs of allograft rejection according to Banff criteria (Banff 0, n=14; Banff borderline, n=2). The median time

between transplantation and allograft biopsy was 15 days (range, 4–532 days).

A small number of peritubular capillaries and larger veins demonstrated a positive DARC signal on endothelial cells in the control group (Fig. 1C and Fig. 2A). Endothelial cells of glomerular capillaries (Fig. 1C and Fig. 2A), arterioles (Fig. 1D), and arteries demonstrated no DARC protein expression. In the control biopsy specimens, the mean number of DARC-positive vessels ranged between 0.3 and 6.8 per HPF, with a mean of 2.6 ± 0.5 (Fig. 3).

DARC Expression in Acute Cellular Rejection

Biopsy specimens from eight patients were included with acute cellular allograft rejection (Banff 1) in the absence of C4d deposition in PTC. The median time between transplantation and allograft biopsy was 10 days (range, 5–20 days). A prominent association was found between sites of DARC-positive PTC and sites of inflammatory cell infiltration (Fig. 1B). No expression of DARC was found on glomerular or arterial endothelium. The number of DARC-positive PTC was higher in this group as compared with the control group ($P < 0.05$) (Fig. 3). The number of DARC-positive vessels ranged between 2.1 and 22.3 per HPF, with a mean of 9.7 ± 2.4 per HPF.

DARC Expression in Humoral Allograft Rejection

Eleven biopsy specimens were included with deposition of C4d in peritubular capillaries but without signs of cellular allograft rejection (according to Banff criteria). The histologic features found in this group were acute tubular necrosis in seven biopsy specimens. Two biopsy specimens did not demonstrate significant abnormalities. Furthermore, two biopsy

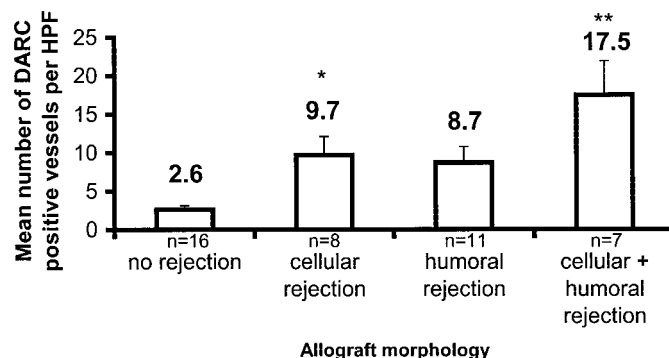


FIGURE 3. Mean number of DARC-positive vessels per HPF (* $P < 0.05$ vs. no rejection, ** $P < 0.01$ vs. no rejection, error bars show SEM).

specimens showed borderline lesions (according to Banff 97) in combination with transplant glomerulitis and thrombotic microangiopathy, respectively. The median time between transplantation and allograft biopsy was 11 days (range, 5–28 days). Similar to the group with Banff 1 rejection, a high number of DARC-positive PTC was found in these patients (with “pure” humoral allograft rejection, Fig. 2C, compare with Fig. 2B). DARC-positive peritubular capillaries were commonly filled with inflammatory cells (Fig. 1E). The mean number of DARC-positive vessels ranged between 0.2 and 22.6 per HPF, with a mean number of 8.7 ± 2.1 per HPF (Fig. 3).

Seven biopsy specimens were included with both C4d deposition and cellular allograft rejection. The median time between transplantation and allograft biopsy was 32 days (range, 6–496 days). The highest numbers of DARC-positive vessels were found in this group (Fig. 2D and Fig. 3). Granulocytes were occasionally detected attached to DARC-positive endothelium (Fig. 1F). In a single case with signs of both humoral and cellular rejection, a positive DARC signal was found on a small number of glomerular capillaries from a single glomerulus, in addition to the staining of PTC. The number of DARC-positive vessels ranged between 1.7 and 32.7 per HPF, with a mean number of 17.5 ± 4.4 ($P < 0.01$ vs. control). Only a single biopsy specimen in this group was found with a mean number of DARC-positive vessels within the range of the normal controls.

DISCUSSION

Acute rejection episodes during the first year after renal transplantation are of significant impact for long-term renal survival, and the incidence has been reduced by modern regimens of immunosuppression (26). Although the significance of humoral allograft rejection mechanisms was proposed almost a decade ago, the prognostic impact and the therapeutic impact have only recently been defined in further detail (19–21). Deposition of the complement split-product C4d in PTC in combination with an accumulation of inflammatory cells, at times neutrophils, is a typical feature of acute humoral renal allograft rejection (19, 27). DARC is expressed at the same site at which C4d is deposited (3, 15, 18). The deposition of C4d in PTC in combination with an accumulation of inflammatory cells (all of which are target cells for DARC ligands) raises the question of whether DARC

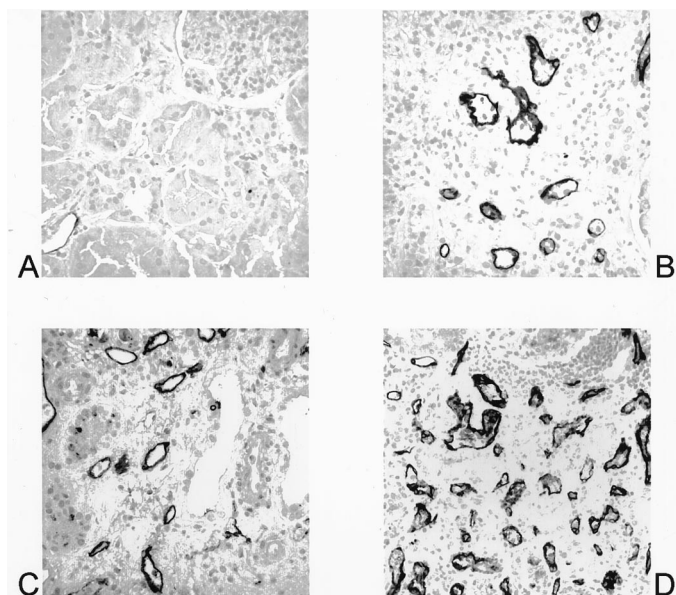


FIGURE 2. Immunohistochemistry performed with the monoclonal anti-DARC (2C3) antibody. Comparison of DARC expression in biopsy specimens without signs of allograft rejection (A), with Banff 1 cellular allograft rejection (B), with acute humoral allograft rejection (C), and with signs of humoral and cellular allograft rejection (D) (magnification $\times 200$).

might be involved in the pathogenesis of humoral allograft rejection. This hypothesis was addressed in the present study by defining the morphologic distribution of DARC in humoral rejection in comparison with cellular rejection.

Included were biopsy specimens from four patient groups, that is, biopsy specimens without signs of rejection, biopsy specimens with a single form of rejection (cellular or humoral), and biopsy specimens with both cellular and humoral rejection. The number of DARC-positive vessels was similar in patients with allograft dysfunction in association with C4d deposition in PTC, in the absence of signs of cellular allograft rejection, as compared with patients with cellular rejection. Therefore, both forms of allograft rejection can result in an induction of DARC on peritubular capillaries, with a similar absolute magnitude.

From these results, the following conclusions may be drawn: DARC is present on a high number of PTC in both humoral allograft rejection and acute cellular rejection, and might be involved in the local accumulation of inflammatory cells. The finding of DARC induction on C4d-positive peritubular capillaries supports the notion that C4d deposition and DARC are markers of a site of endothelial activation or injury. The importance of PTC has been highlighted previously as the site of inflammatory cell extravasation using binding assays for lymphocytes on frozen allograft sections (7). Furthermore, evidence for activation of PTC leading to endothelial cell thickening, with loss of fenestration, in combination with increased adherence and passage of lymphocytes and monocytes has been demonstrated (28). DARC can bind a number of chemokines active on the most commonly found inflammatory cells infiltrating renal allografts. The authors previously demonstrated an association between induction of DARC and acute renal allograft rejection according to Banff criteria. Furthermore, an association was found between inflammatory cell infiltration with the number of DARC-positive vessels (18). The additive effect of cellular and humoral rejection on the expression of DARC found in this study may indicate different pathways of DARC induction in these forms of allograft rejection. Although the two forms of allograft injury, humoral and cellular, are different in the morphologic presentation and in the prognostic impact, the PTC seem to be a site of activation in both forms.

The induction of DARC in the two settings might be because of a similar proinflammatory milieu that results in a comparable induction of DARC. The additive effect argues against this hypothesis but favors different pathways of induction. The additive effect on DARC expression might also be a hint for different types of vessels that might be involved during different forms of rejection. Different subsets of PTC have previously not been described, and there was no apparent difference in the pattern of DARC-positive PTC between both forms of rejection.

The functional role of endothelial DARC for cell recruitment is still obscure. DARC might help to present chemokines on the endothelial surface to leukocytes expressing the corresponding receptors. A second possibility is that binding of chemokines to DARC helps to keep up a chemokine gradient at the site of cell extravasation. A third alternative is that chemokines bound to DARC lose their chemoattractive potential. This function has been postulated for DARC expressed on red blood cells, and DARC might be able to pursue different functions according to the spatial and the temporal

context. Although the functional role of DARC during renal allograft rejection remains obscure, the induction on PTC in all types of human renal allograft rejection, demonstrated for humoral allograft rejection in this study for the first time, implies a role in the recruitment of inflammatory cells. Until recently, the functional roles of DARC have not been addressed, in part because of the absence of experimental *in vitro* and *in vivo* systems adequate to address these questions. Mice deficient in DARC have now been established by different groups, and both proinflammatory and anti-inflammatory roles have been described in these mice in different disease models (29, 30). With the availability of DARC-deficient mice on a defined genetic background, detailed studies on DARC function are now possible by means of transplantation experiments of DARC-positive (respectively negative) allografts into DARC-negative (respectively positive) recipients. It is hoped that these might help to define the functional significance of DARC induction during human allograft rejection in the near future.

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TRANSPLANTATION

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POSTTRANSPLANTATION PRODUCTION OF DONOR HLA-SPECIFIC ANTIBODIES AS A PREDICTOR OF RENAL TRANSPLANT OUTCOME¹

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Background. This study aimed to determine whether the production, in renal transplant recipients, of antibodies directed against donor HLA mismatches is predictive of transplant failure.

Methods. The failure study group comprised 112 adult recipients of primary renal transplants who had re-entered the transplant waiting list after failure of the first graft. A control group of 123 recipients with functioning transplants was selected from transplantations performed during the same time period, in which patients had equivalent HLA matching and immunosuppression and a minimum of 5 years of follow-up. Sera taken before transplantation and at 1, 3, and 6 months and annually after transplantation were tested by enzyme-linked immunoabsorbent assay

(ELISA) for the presence of HLA class I- and class II-specific antibodies. Antibody specificity was defined by a combination of cytotoxicity, ELISA, and flow cytometry techniques to determine whether the antibodies were directed against donor mismatches.

Results. All recipients were negative for donor HLA-specific antibodies before transplantation. After transplantation, 57 (50.9%) of the 112 patients in the failure group produced donor HLA-specific antibodies compared with 2 (1.6%) of the 123 controls ($P < 0.0001$; odds ratio [OR] = 64.98; confidence interval [CI], 14.78–399.51). For 60% of the donor-specific antibody-positive patients, antibodies were detected before transplant failure. In 17 cases, these were class I specific; in 14 cases, class II specific; and in 3 cases, specific for both class I and II.

Conclusions. This study has demonstrated that the production of posttransplantation antibodies directed against donor HLA-A, -B, -Cw, -DR, and -DQ mismatches are all strongly predictive of transplant failure.

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Despite the use of modern immunosuppressive therapy, adaptive immune responses to grafted organs are still the major impediment to successful transplantation. The clinical relevance of preformed donor-specific HLA antibodies was demonstrated as early as 1966 and resulted in the introduction of the pretransplantation crossmatch (1). Later studies showed that the presence of HLA-specific antibodies after transplantation was associated with poor kidney function.