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ORIGINAL ARTICLE

Capillary deposition of the complement fragment C4d in cardiac allograft biopsies is associated with allograft vasculopathy

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Summary

Cardiac allograft vasculopathy (CAV) is a long-term threat in heart transplant recipients and its exact pathogenesis remains to be established. As complement activation contributes to early and late allograft dysfunction, we hypothesized that deposition of the complement fragment, C4d, in capillaries of cardiac allograft biopsies may be associated with CAV. A polyclonal anti-C4d antibody was used for immunohistochemistry on endomyocardial biopsies obtained from heart transplant recipients during the first year post-transplantation. CAV was assessed by intracoronary ultrasound performed at 1-year post-transplantation. We were able to show that CAV is highly associated with C4d deposition in capillaries of cardiac allografts and that serial C4d studies may predict development of CAV at 1-year post-transplantation.

Introduction

Cardiac allograft vasculopathy (CAV) is a long-term threat in heart transplant recipients. Intimal thickening progresses most rapidly during the first year after transplantation and is detectable by intracoronary ultrasound (ICUS) in up to 75% of patients by the end of the first year [1]. Although the disease appears to be primarily immune-mediated, the exact pathogenesis remains to be established. The complement system, which can be activated by both antigen-specific and antigen-nonspecific events [2] has been implicated as a contributing factor to early and late allograft dysfunction in cardiac transplantation [3]. In particular, deposition of the complement split product C4d in endomyocardial biopsies (EMBs) appears highly associated with graft loss [4].

Our study investigates the hypothesis that capillary deposition of C4d in cardiac allograft biopsies of human heart transplant recipients is associated with the development of CAV.

Methods

Patients

Heart transplant recipients in whom serial EMBs and an ICUS study for accurate assessment of CAV have been performed were included into the study. A total of 64 right ventricular (EMBs) were obtained from 17 heart transplant recipients during routine rejection monitoring at 2, 7, 27 and 52 weeks post-transplantation. ICUS was performed at the time of the last EMB. Indications for heart transplantation were ischemic ($n = 11$) and dilated

cardiomyopathy ($n = 6$). No patient revealed high immunologic risk, panel reactive antibodies (PRA) > 30%) or was treated with an assist device pretransplantation. Postoperative complement-dependent lymphocytotoxic crossmatch was negative in all patients. All patients received prophylactic therapy with antithymocyte globin and standard immunosuppression with cyclosporin A ($n = 16$) or FK506 ($n = 1$), azathioprine ($n = 2$) or mycophenolate mofetil ($n = 15$), and prednisone ($n = 14$).

Endomyocardial biopsies

Four to five myocardial biopsies were obtained per patient and fixed in formalin. Grading of acute cellular rejection was performed according to the modified ISHLT criteria [5]. Grading for humoral rejection was based on the suggestions by Olson *et al.* [6]. A novel polyclonal anti-C4d antibody (C4dpAb; Biomedica, Vienna, Austria) was used for detection of C4d. We used indirect immunoperoxidase staining for immunohistochemical detection of C4d on formalin-fixed, paraffin-embedded sections as previously described [7]. The intensity of endothelial C4d staining was classified $C4d^-$ in the absence of endothelial staining, and $C4d^+$ when only a few capillaries stained positive; $C4d^{++}$ denoted staining of more than 50% of all capillaries or partial staining of most of the capillaries, and $C4d^{+++}$ markedly deposition of C4d in all capillaries. Biopsies of 15 explanted age-matched donor hearts, which had been rejected for various reasons, served as controls.

Intracoronary ultrasound studies

The ICUS was performed in the left anterior descending artery with a 3.2F/40 MHz ultrasound transducer (CVIS/Boston Scientific, Fremont, CA, USA). The most distal position was documented with cineangiography and a continuous slow manual pullback was performed. Videoimages were analyzed by computerized planimetry using 4–7 (mean 5.2) evenly spaced positions during the ICUS pullback. The following measurements were obtained: (i) total vessel area (VA); (ii) lumen area (LA); (iii) plaque area (PA = VA − LA). Diagnosis of CAV was based on mean intimal index: PA÷VA. An intimal index ≥ 0.25, was considered significant CAV.

Statistical analysis

Results are presented as mean ± SD. Pearson's or Spearman's correlation coefficients were determined to assess associations of immunologic and nonimmunologic risk factors with intimal index and C4d staining results. C4d staining results and intimal index were compared by a mixed effects model based on the number of biopsies to account for dependencies within the same patients. ANOVA, *t*-test, and chi-square test or Fisher's exact test were employed for comparisons based on single measurements per patient, as appropriate.

Results

The EMBs of the study patients were subdivided into four groups according to the intensity of endothelial C4d staining (C4d 0, $C4d^+$, $C4d^{++}$, $C4d^{+++}$). Patients were allocated to each group according to their maximal score in any biopsy investigated. Table 1 shows the distribution of intimal index and parameters with potential impact on the development of CAV among C4d groups. No C4d staining was found in 15 control biopsies.

Intimal index per patient varied from 0.14 to 0.54. Capillary C4d deposition was highly associated with intimal index in a mixed effects model ($r = 0.41$; $P = 0.001$). In this series of patients, the only other parameter that correlated with intimal index was donor age ($r = 0.68$; $P < 0.001$). No association with intimal index was seen for human leukocyte antigen (HLA)-A, -B, -DR mismatch, number of cellular rejections >ISHLT 1b, recipient age, cold ischemia time, etiology of cardiomyopathy, or immunosuppressive therapy.

To analyze the predictive value of immunohistochemistry, capillary C4d deposition was subdivided into positive ($C4d^{++}/C4d^{+++}$) and negative ($C4d^-/C4d^+$) results. Sixteen of 64 biopsies (25%) were found to be positive. Nine of these 16 positive biopsies were obtained after the third month post-transplantation (Fig. 1). To assure high

Table 1. Distribution of intimal index as well as immunologic and nonimmunologic risk factors among C4d groups.

	$C4d^{+++}$	$C4d^{++}$	$C4d^+$	C4d 0
EMBs	2	14	18	30
Patients	2	7	5	3
Intimal index	0.29 ± 0.21	0.28 ± 0.16	0.20 ± 0.04	0.18 ± 0.02
Cold ischemia time (min)	252 ± 13	173 ± 52	192 ± 26	165 ± 72
HLA-A, -B, -DR mismatch	4.0 ± 0.0	4.4 ± 1.0	4.6 ± 0.9	4.3 ± 0.6
Number of cellular rejection >ISHLT 1b in year 1	0	3/7	0/5	1/3

EMB, endomyocardial biopsy; HLA, human leukocyte antigen.

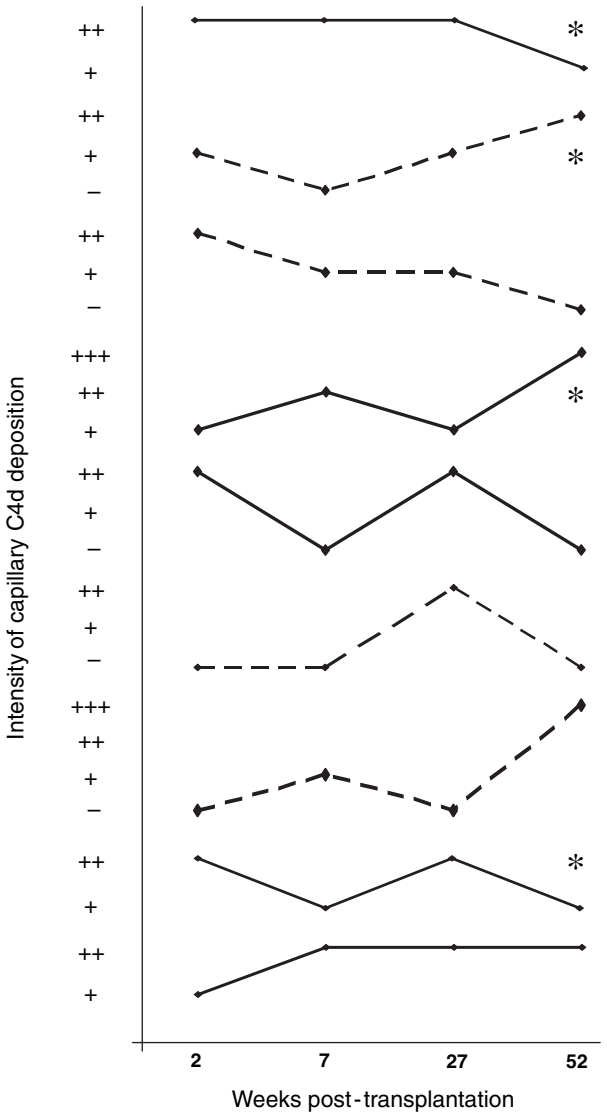

Figure 1 Kinetics of capillary C4d deposition in nine patients with $C4d^{++}/C4d^{+++}$ deposits in any of the biopsies obtained during the first year post-transplantation. Patients (denoted by solid lines) were classified C4d-positive if at least two biopsies were graded positive. Patients, who developed significant cardiac allograft vasculopathy (CAV) at 1-year post-transplantation are marked with an asterisks (*). See text for further explanation.

specificity, patients were classified as C4d-positive if at least two biopsies during the first year were graded positive (Table 2). Intimal index was higher in C4d-positive recipients when compared with C4d-negatives (0.33 ± 0.17 vs. 0.19 ± 0.06; $P = 0.002$). Three of five C4d-positive recipients versus one of 12 C4d-negative recipients revealed significant CAV. The specificity for the association of C4d-positive status and CAV was 85% (54–98), the sensitivity 75% (19–100), the positive predictive value was 60% (15–95), and negative predictive value was 92% (62–100) (95% CI). All patients with CAV showed at least one biopsy graded $C4d^{++}$, but clusters of positive biopsies were found only in two patients (Fig. 1). No recipient graded C4d-negative ($C4d^{-}/C4d^{+}$) in any of the obtained biopsies presented significant CAV ($n = 8$).

No association was found between the number of cellular rejection episodes >ISHLT 1b and HLA-A, -B, -DR mismatches, or with cold ischemia time (results not shown). Of note, conventional histopathologic evidence of humoral rejection was found neither in C4d-positive nor in C4d-negative biopsies. Left ventricular ejection fraction at 1-year post-transplantation did not differ between C4d-positive and C4d-negative patients (64% vs. 65%).

Discussion

The main findings of this study are (i) that CAV is highly associated with the activation of the complement system in cardiac allografts, and (ii) that immunohistochemical evaluation of cardiac allograft biopsies can identify patients who are at risk for the development of CAV at 1-year follow up.

The CAV is thought to be the result of inflammatory and proliferative responses to subclinical graft coronary endothelial injury. Evidence is accumulating for the pervasive role of complement activation in these processes [2]. In our study, capillary deposition of C4d in cardiac allograft biopsies during the first year post-transplantation was highly associated with the development of CAV at 1-year follow up. The concept that complement activation is involved in the development of CAV is also supported by a recent animal study by Qian *et al.* [8]. Further evidence arises from the relationship between a prothrombogenic microvasculature and the development of CAV. Behr *et al.* have demonstrated an association between C4d deposition and fibrin deposition in capillaries of human cardiac allografts [4]. A second study showed correlation of fibrin deposition with subsequent CAV and graft failure [9]. In Behr's study [4] C4d deposition was highly associated with graft loss.

Patients in our study were subdivided into two groups according to C4d staining results. Intimal index as a measure of CAV was higher in C4d-positive than in C4d-negative patients. C4d-positive status in our study patients would have detected 60% of those who developed significant CAV (negative predictive value of 92%). Hence, repeat staining for C4d in cardiac allograft biopsies during the first year post-transplantation provides a valuable tool for the prediction of subsequent CAV. Patients with recurrent and positive staining results ($C4d^{++}/C4d^{+++}$) are at high risk for the development of

Table 2. Characteristics of patients graded C4d-positive and C4d-negative.

	All	$C4d^+$	$C4d^-$	*P*-value
Patients	17	5	12	
EMBs	3.3 ± 0.7	3.4 ± 0.55	3.25 ± 0.75	0.7
Intimal index	0.24 ± 0.12	0.33 ± 0.17	0.19 ± 0.06	0.002
Diagnosis IHD	11	3	8	1.0
Recipient age	55 ± 7	50 ± 8	57 ± 5	0.05
Women recipients	3/17	1/5	2/12	1.0
Sex mismatch	3/17	1	2	1.0
Donor age	36 ± 11	47 ± 6	32 ± 10	0.007
Cold ischemia time	186 ± 50	189 ± 71	185 ± 43	0.9
HLA-A, -B, -DR mismatch, positions	4.4 ± 0.8	4.25 ± 0.5	4.5 ± 0.9	0.5
Number of cellular rejection >ISHLT 1b in year 1	4/17	2/5	2/12	0.5

EMB, endomyocardial biopsy; HLA, human leukocyte antigen; IHD, ischemic heart disease.

CAV whereas consistently negative results ($C4d^-/C4d^+$) indicate a low risk.

It is of note that Vallhonrat *et al.* did not find a correlation between plasma levels of C4d and CAV, indicating the necessity for immunocytochemical procedures [10]. This can be explained by the fact that C4d is covalently bound to endothelium via a highly reactive thioester-group and thus is resistant to shedding. However, staining for C4d may be performed on routine EMB specimens obtained to monitor rejection and thus does not require additional invasive procedures.

Several mechanisms have been implicated in complement activation post-transplantation. Capillary deposition of C4d complement fragment was previously shown to identify humoral antigraft reaction in human heart and kidney transplants and alloantibody activity in cardiac allografts have been discussed repeatedly in the pathogenesis of CAV [4,7].

Local activation of the complement system in cardiac allografts may thus represent the missing link between recurrent antibody-mediated alloreactions, vascular inflammation, and subsequent CAV. Our finding may also explain why patients who demonstrate a recurring pattern of microvascular rejection have significantly worse survival rates [11]. The fact that a diagnosis of humoral rejection in our study was based entirely upon detection of C4d deposition is well in line with the results of Behr *et al.* [4]. In their study, C1q, C3c, and immunoglobulin M (IgM) staining results showed no correlation with clinical outcome. Furthermore, no correlation was seen between C4d staining and histologic markers of humoral rejection indicating the limited value of these techniques. In addition, we did not see positive C4d staining results in control biopsies suggesting a high specificity of this marker. It cannot be excluded that investigating monocyte/macrophage infiltration by immunohistochemistry (e.g. CD68) may have revealed histologic pattern of humoral rejection in C4d-positive patients. Also, the fact that postoperative complement-dependent lymphcytotoxic cross-match was negative in C4d-positive patients does not conflict with the proposed immune mechanism. It excludes neither the presence of anti-HLA class II antibodies nor *de novo* production of antidonor antibodies after transplantation.

It is striking that in contrast to previous studies, C4d expression in our patients was neither associated with hemodynamic compromise nor with allograft dysfunction at 1-year post-transplantation. However, these findings are in line with recent results from Michaels *et al.*, also indicating no difference in the occurrence rate of CAV in patients with humoral rejection with or without hemodynamic compromise [12].

Previous studies have shown activation of the endothelium and the complement system early in the course post-transplantation which was attributed mostly to ischemia-reperfusion injury [3]. Instead, we did not see a timely fashion of high C4d expression during the first year. No association was found for the intensity of C4d deposition with cold ischemia time either. Including more 'early' biopsies into the study may have confirmed this correlation. However, it is conceivably that only long-term activation of the complement system because of recurrent or prolonged antibody-mediated alloreactions rather than short-term activation associated with ischemia-reperfusion injury will eventually translate into development of CAV.

Based on our study, we suggest an association between CAV and activation of the complement system in human cardiac allograft recipients. Recurrent or ongoing antibody-mediated alloreactions may trigger this process. Immunohistochemical assessment of C4d deposition in cardiac allograft biopsies may provide a useful tool to identify patients who are likely to develop relevant CAV. These patients may require additional immunosuppressive therapy targeted against the humoral immune system or alternative therapy such as complement.

Larger prospective studies are needed to confirm the contribution of C4d to the development of CAV and its predictive value.

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