ELSEVIER

Measurement of Human Erythrocyte C4d to Erythrocyte Complement Receptor 1 Ratio in Cardiac Transplant Recipients With Acute Symptomatic Allograft Failure

K.C. Lee, C.Y. Chang, Y.C. Chuang, S.H. Sue, T.W. Chu, R.J. Chen, S.H. Chen, J. Wei, and C.H. Chen

ABSTRACT

Background. Complement activation has been recognized as a contributing factor to cardiac allograft dysfunction. Combined measurement of erythrocyte C4d (E-C4d) and complement receptor 1 (E-CR1) are potential biomarkers to monitor complement activity in patients with autoimmune diseases. We conducted a prospective study using CR1-2B11 monoclonal antibody to detect the E-C4d to E-CR1 ratio among our cardiac transplant recipients with acute symptomatic allograft failure.

Materials and methods. Eight recipients with acute cardiac allograft failure and 72 healthy controls were included in this study. Levels of E-C4d and E-CR1 were measured by indirect immunofluorescence and flow cytometry. The results were utilized to determine the association between patient C4d staining, histological features, and clinical outcomes.

Results. Eight patients with nine episodes of sudden onset of graft failure and suspected antibody-mediated rejection (AMR) were included in this study. One patient who received emergent mechanical circulatory support was treated with plasmapheresis for his unstable hemodynamic status. The mean pretreatment left ventricular ejection fraction was 30.3%. No histological study demonstrated cellular rejection or AMR in any patient. There were two patients with positive C4d immunostaining. Three patients had four episodes of acute rejection with sudden death at home. The mean E-C4d/E-CR1 ratio in the study group ($n = 9$) was 0.22 ± 0.07, and 0.12 ± 0.10 in the control group ($n = 72$). As comparing both groups, we found the ratios were significant higher in the study group ($P = .0003$).

Conclusions. Measurement of the E-C4d/E-CR1 ratio may be a noninvasive method for detecting acute rejection after cardiac transplantation.

CARDIAC TRANSPLANTATION is an effective treatment for end-stage heart failure. However, acute cardiac allograft rejection can result in a poor prognosis or even a patient's sudden death.[1] With advances in immunosuppressive agents, the diagnosis and treatment for cellular rejection has become more effective. But for the diagnosis and treatment of antibody-mediated rejection (AMR) are still obscure.[2] The histological features that allow us to identify this type of rejection on endomyocardial biopsies (EMB) have been defined in the ISHLT-WF2004. However, a recent report suggested that the sensitivity of the histological criteria is too low to serve as a screening parameter for AMR; the authors recommended the addition of immunostaining to screen for the presence of AMR.[3]

Complement activation has recently been considered to be a factor contributing to early and late graft failure in cardiac transplantation. Several possible markers of AMR have been investigated, especially C4d, the cleavage product of C4. In

From the Heart Center (K.C.L., C.Y.C., Y.C.C., S.H.S., R.J.C., S.H.C., J.W.), Cheng-Hsin General Hospital, Taipei, Taiwan; Center of General Education (K.C.L.), National Taipei College of Nursing, Taipei, Taiwan; Division of Rheumatology, Immunology and Allergy (C.H.C.), Tri-Service General Hospital and National Defense Medical Center, Taipei, Taiwan; and Division of Cardiovascular Surgery (T.W.C.), Lotung Pohai Hospital, Yi-Lan, Taiwan.

Address reprint requests to Chen Hung Chen, Division of Rheumatology, Immunology and Allergy, 325, Section 2, Cheng Gong Rd, Neihu 114, Taipei, Taiwan. E-mail: ch9066@chgh.org.tw

0041-1345/08/$–see front matter
doi:10.1016/j.transproceed.2008.08.027

renal and cardiac allografts, complement fragments C4d deposit in the microvasculature, a marker for AMR.[4–8]

For decades the phenomenon of complement activation has been observed in patients with autoimmune diseases. The proteolytic fragments of complement C4, generated during activation of the classical pathway, are present on the surfaces of normal erythrocytes. Abnormalities in complement activation and clearance of immune complexes by erythrocytes are fundamental to the pathogenesis of systemic lupus erythematosus (SLE). These abnormalities include reduced levels of CR1 on erythrocytes, deficiency in components of the classical pathway including C4, and saturation of CR1 by preexistent immune complexes.[9–13]

Based upon these observations, we supposed that abnormalities in complements activation specific to SLE may be reflected by molecular events involving both receptors and ligands on erythrocyte surfaces. Identification of such abnormal patterns may be useful for a noninvasive diagnosis of AMR in patients with cardiac allograft dysfunction. Therefore, we performed a prospective study in recipients with acute cardiac allograft dysfunction to detect the erythrocyte C4d (E-C4d) to erythrocyte complement receptor 1 (E-CR1) ratio using CR1-2B11 monoclonal antibody in addition to routine histological studies and immunostaining.

MATERIALS AND METHODS

From July 1988 to November 2007, there were 286 orthotopic heart transplantations (HTx) performed in our institution. Before HTx, all patients were referred to our immunologist to exclude the presence or high likelihood of autoimmune disease. Azathioprine (Aza; 1 mg/kg) was administered 1 hour before the operation. Methylprednisolone (500 mg) was infused before release of the aortic cross-clamp, and another 500 mg in the intensive care unit. After surgery, we used rabbit antithymacyte globulin (ATG) for induction therapy (the prophylactic dose = 1.25 mg/kg per day) for 2 to 3 days with dose adjustment by daily flow cytometry to determine T lymphocytes, as well as enumerated white blood cells (WBC) and platelets. Oral Aza, given at the initial dose of 1 to 3 mg/kg per day and then the steady-state dose of 0.5 to 2.0 mg/kg per day, was adjusted to maintain the WBC count between 4000 and 9000/mm^3. Prednisolone (0.3 to 0.5 mg/kg per day), which began on the second posttransplant day, was tapered to 5 to 10 mg/d 1 month later. Oral cyclosporine (CyA), which also began on the second day, was adjusted according to recipient renal function and blood CyA trough and peak levels. During the first 3 months, the trough CyA level was maintained at 350 to 450 ng/mL and then shifted to 100 to 250 ng/mL 6 months later. If CyA was relatively contraindicated, then oral tacrolimus was prescribed at 0.15 to 0.3 mg/kg per day and then the dose was adjusted to keep the initial trough level around 10 to 20 ng/mL and the maintenance level at 5 to 10 ng/mL. Since 1997, we have used mycophenolate mofetil (MMF) at the dose of 1 to 2 g/d to replace Aza. Its dose has also been adjusted according the recipient WBC count. Prophylactic anti-cytomegalovirus (CMV) therapy with intravenous ganciclovir was administered for 14 days, at a dose based on the recipient's renal function. After HTx, the recipient underwent EMB weekly for four times, biweekly for eight times, bimonthly for 6 months, and yearly thereafter. If pathological results of EMB showed grade II rejection (ISHLT-WF1990) or more, the recipient would be hospitalized for the pulse therapy with methylprednisolone. Strict diet control and counter measure medications were used to treat hyperlipidemia, diabetes, and hypertension. A coronary angiogram was performed annually to assess recipient cardiac allograft vasculopathy. If any patient developed a major adverse cardiac event, such as heart failure, arrhythmia, or significant electrocardiograph change, EMB and coronary angiogram were performed as soon as possible for assessment. Since 2003, panel reactive antibody (PRA) levels and crossmatching were routinely performed in our recipients. The doses of immunosuppressants were adjusted according to the recipient PRA and crossmatch.

From August 2007 to April 2008, there were nine patients who experienced a sudden onset of severe allograft dysfunction without any sign of acute cellular rejection or myocardial ischemia; they were admitted to our intensive care unit for therapy. Eight patients with nine events were included in this study. One female patient was excluded because her subsequent diagnosis of cardiac amyloidosis was established by re-HTx. All patients received emergent coronary angiograms and EMB upon admission. They all experienced blood sampling for this study before any treatment for allograft dysfunction. Written informed consent was obtained from our patients before blood sampling; informed consent form and the study protocol had been approved by our Institutional Review Board.

All patients with suspected acute rejection episodes were treated with inotropic agents, methylprednisolone pulse therapy (1 g/d for 3 days), and increased dosages of MMF immediately. Infusion of ATG was indicated if the pulse therapy showed a suboptimal immediate response. If these treatments failed to show any satisfactory response, then the patients received plasmapheresis until graft recovery. Mechanical circulatory supports, such as intra-aortic balloon pumping or extracorporeal membrane oxygenation, were used in patients with profound shock and unstable hemodynamic status. Afterward, their immunosuppressive regimens were modified by increased MMF doses higher target trough levels of CyA or replacement by tacrolimus.

Healthy Controls

A total of 72 subjects of healthy controls were recruited through local advertisements posted in areas around the Tri-Service General Hospital. The demographic data, including age, race, and gender, as well as their past medical history were collected for these healthy controls, to confirm their disease-free status.

Flow Cytometric Characterization of Erythrocytes

A 3-mL sample of blood obtained from each study participant and collected in a Vacutainer tube (Becton Dickinson, Franklin Lakes, NJ, USA), containing ethylenediamine-tetraacetic acid as an anticoagulant, was used for experiments on the day of collection. Whole blood was diluted in phosphate-buffered saline (PBS) containing 1% bovine calf serum. The erythrocytes were pelleted, washed with PBS containing bovine calf serum, and aliquoted for antibody staining. Mouse monoclonal antibodies specific for CR1 (CR1-2B11),[12] human C4d (reactive with all C4d-containing fragments of C4; Quidel, San Diego, Calif, USA), or the isotype-matched control TS1 were added to the erythrocytes at a concentration of 5 μg/mL. Fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin-specific polyclonal antibody (Multiple Adsorption; BD Pharmingen, NJ, USA) was added at a concentration of 0.5 mg/mL. The cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry System, San Jose, Calif, USA). The erythrocytes were electronically gated based on forward and side scatter properties to include only single cells.

Surface expressions of E-CR1 and E-C4d on the gated cells were expressed as specific mean fluorescence intensity (MFI). The E-C4d/E-CR1 ratio was calculated using the following equation: (E-C4d MFI − isotype-matched control MFI) ÷ (E-CR1 MFI − isotype-matched control MFI).

Statistical Analysis

Nominal and ordinal variables were tabulated for frequency distributions. Scale variables were summarized as mean values ± standard deviations or medians (range). Mann-Whitney test was used to compare the E-C4d levels between the two study groups and their E-CR1 levels. Statistical significance was set at P values below .05.

RESULTS

Based on the above criteria, there were nine suspected episodes of AMR in eight patients. One patient (No. 8) experienced two episodes of acute allograft dysfunction. The pre-HTx characteristics of our study group were tabulated in Table 1. There was one woman and seven men in this study with an overall mean age of transplantation of 33.4 years. For the reason described above,[14] patient No. 8 had a longer ischemia time during HTx. Because of his poor medical compliance, the patient also had more events of CMV infection and acute cellular rejection. There was no high PRA or positive crossmatching in our study group (n = 7). The mean time to suspected AMR after HTx was 27.3 months (range: 1–52 months).

The mean left ventricular ejection fraction in acute graft failure was 30.3% (Table 2). All patients received the support of inotropes; mechanical circulatory support was used in patient No. 8 for his sudden onset of hemodynamic collapse during the treatment of graft failure. All patients received steroid pulse therapy. ATG was infused in three patients, and plasmapheresis in patient No. 8. The histological study demonstrated no obvious acute cellular rejection or AMR. Two patients showed positive immunostaining of C4d, and the two other patients also had positive C4d staining during their outpatient clinic visits. All eight patients recovered and were discharged after successful treatment. However, during their follow-up, three patients suddenly died at home. One patient suffered another AMR with positive C4d staining on the time of writing this article. He received 10 sessions of plasmapheresis and his cardiac function unfortunately only showed partial recovery.

The mean E-C4d MFI was 3.62 ± 0.55 in the study group (n = 9), and 3.39 ± 0.78 in the control group (n = 72); (P = .1111) the results of E-C4d/E-CR1 ratios, it was 0.22 ± 0.07 in the study group, and 0.11 ± 0.10 in the control group (P = .0003). If we defined the study group by the diseased status, then we found the E-C4d/E-CR1 ratio was a significant predictor for the diseased status (odds ratio = 2560, P = .010). However, when we divided our study patients into groups with good (patients No. 1, 2, 3, and 5) and poor (patients No. 4, 6, 7, 8-1, and 8-2) prognoses, we found the ratio could not predict the prognosis of our patients with AMR (P = .2207).

DISCUSSION

Immunosuppression largely modulates the T-cell component, preventing cellular rejection due to the sensitivity of the T-cell biological cycle to CyA or tacrolimus. These drugs interrupt cell proliferation. The function of B cells and, hence, AMR, then becomes a priority.[15] Last, it was believed that most AMR occurs early, but now it is known that AMR can, and most commonly does, occur months and even years after transplantation.[16,17] Our study showed that AMR can develop from 1 to 52 months after HTx. Although AMR is well recognized and accepted in the field of renal transplantation, it has not gained universal consensus in the cardiac field, despite numerous studies from cardiac-related literature of basic science, immunology, clinical medicine, and pathology.[18] The antibodies used in the evaluation of immunofluorescence changed over time. Positive immunofluorescence of IgG, IgM, C3, C1q, and fibrinogen do not always correlate with hemodynamic compromise or development of cardiac allograft vasculopathy, which has resulted in declining use of these tests.[7] Since the sensitivity of histological criteria is too low to serve as a screening parameters for AMR, most reports recommend the addition of immunostaining to screen for the presence of AMR.[15–17] Because complement may be activated during procedures, such as extracorporeal circulation during

Table 1. Characteristics of Acute Allograft Failure in the Eight Heart Transplant Patients

Patient No.	Age (y)/ Gender	Time after HTx (mo)	Donor Age (y)	Ischemia Time (min)	Preoperative PRA (%)	Crossmatching	Episodes of CMV Infection	Episodes of Acute Cellular Rejection (□Grade II)	Coronary Lesion or Cardiac Allograft Vasculopathy
1	28/M	52	22	113	NA	NA	0	0	No
2	12/F	47	25	241	4.0	−	2	0	No
3	32/M	42	33	216	2.2	−	0	2	No
4	52/M	8	43	141	1.1	−	0	0	Yes†
5	21/M	10	44	225	2.4	−	1	0	No
6	54/M	7	44	250	5.3	−	0	0	No
7	54/M	1	49	210	2.5	−	0	0	No
8-1*	14/M	36	47	779	0.9	−	6	4	No
8-2*	14/M	43	47	779	0.9	−	6	4	No

HTx, heart transplantation; PRA, panel-reactive antibody; NA, not available; CMV, cytomegalovirus.
*The same patient had two episodes of acute allograft failure.
†40% stenosis over middle left anterior descending coronary artery.

Table 2. Outcome of Acute Allograft Failure in the Eight Heart Transplant Patients

Patient No.	EF (%) + HS	Immunosuppressive Treatments	Grading of Cellular Rejection*	Histological AMR†	C4d Staining	E-C4d/ E-CR1	Recovery of Graft Function	Current Status
1	30 + I	Pulse therapy	IA	−	−	0.26	Yes	Alive
2	30 + I	Pulse therapy	IB	−	−	0.11	Yes	Alive
3	28 + I	Pulse therapy	IB	−*	25% positive	0.16	Yes	Alive
4	35 + I	Pulse therapy + ATG	IA	−	−	0.24	Yes	SD
5	35 + I	Pulse therapy	IA	−	5% positive	0.23	Yes	Alive
6	35 + I	Pulse therapy	IA	−	−‡	0.18	Yes	Re-AMR
7	35 + I	Pulse therapy + ATG	IB	−	−‡	0.2	Yes	SD
8-1	30 + I	Pulse therapy	IB	−	−	0.34	Yes	SD
8-2	15 + I + IABP + ECMO support	Pulse therapy + ATG + plasmapheresis	IA	−	−	0.30	Yes	SD

EF, ejection fraction; HS, hemodynamic support; I, inotropic agents; IABP, intra-aortic balloon pumping; ECMO, extracorporeal membranous oxygenation; ATG, anti-thymocyte globulin; AMR, antibody-mediated rejection; SD, sudden death.
*As defined in the ISHLT-1990.
†As defined in the ISHLT-2004.
‡Positive C4d staining during subsequent follow-up.

surgery, by ischemia-reperfusion injury, and by induction therapy before transplant with ATG, the mere presence of C4d and/or C3d in capillaries should not be equated with AMR. According to the report of Tan et al,[16] the use of C4d immunostaining alone is not a reliable tool. They suggested that it should include staining for both C4d and C3d; immunostaining for C4d alone can be misleading because about 10% of the patients showed either C4d or C3d deposits alone in capillaries without clinical evidence of allograft dysfunction. However, in our patients with clinical evidence of allograft dysfunction, the incidence of positive immunostaining for C4d was quite low, which meant that the sensitivity and specificity of C4d immunostaining for the diagnosis of AMR needs further investigation. Some studies[19] have measured the plasma level of C4d fragments, concluding that it was not a useful noninvasive method to detect AMR after HTx.

In our study, we also observed that patient E-C4d mean fluorescence intensity did not display a significant difference compared with the control group. E-C4d/E-CR1 ratios increased significantly in the study group. According to the reports of complement activation specific in SLE, we believe that measurement of E-C4d/E-CR1 plasma levels are a more objective method to identify complement activation in patients with AMR after HTx. Nevertheless, from out study, this ratio was not a significant prognostic factor for the AMR among our heart transplant patients, which may be attributed to the limited sample size. Accurate enumeration of cell surface CR1 is also crucial for the detection of complement activation. We believe that it can be improved by using specific mouse monoclonal antibodies (CR1-2B11).[20]

This study is just in the early stage of detection for the E-C4d/E-CR1 ratio in the patients with AMR; there are still study limitations. First, we need to collect more data from the group of cardiac transplant recipients without evidence of rejection for further analysis. Second, we need to examine the change in the E-C4d/E-CR1 ratio among patients who receive treatment and recover from AMR. The final purpose of this study is to clarify the correlation between circulating and histologically depositing complement fragments.

In Conclusion, we concluded that the measurement of the E-C4d/E-CR1 ratio may serve as a noninvasive method to detect acute cardiac allograft dysfunction. However, we need further investigation to clarify the correlation between circulating and histologically depositing complement fragments.

REFERENCES

1. Hunt S, Billingham M: Long-term results of cardiac transplantation. Annu Rev Med 42:437, 1991
2. Michaels PJ, Espejo ML, Kobashigawa J, et al: Humoral rejection in cardiac transplantation: risk factors, hemodynamic consequences and relationship to transplant coronary artery disease. J Heart Lung Transplant 22:58, 2003
3. Hammond ME, Stehlik J, Snow G, et al: Utility of histological parameters in screening for antibody-mediated rejection of the cardiac allograft; a study of 3,170 biopsies. J Heart Lung Transplant 24:2015, 1995
4. Regele H, Exner M, Watschinger B, et al: Endothelial C4d deposition is associated with inferior kidney allograft outcome independently of cellular rejection. Nephrol Dial Transplant 16: 2058, 2001
5. Behr TM, Feucht HE, Richter K, et al: Detection of humoral rejection in human cardiac allografts by assessing the capillary deposition of complement fragment C4d in endomyocardial biopsies. J Heart Lung Transplant 18:904, 1999
6. Bohmig GA, Exner M, Habitcht A, et al: Capillary C4d deposition in kidney allografts: a specific marker of alloantibody-dependent graft injury. Am J Nephrol 13:1091, 2002
7. Bonnaud EN, Lewis NP, Masek MA, et al: Reliability and usefulness of immunofluorescence in heart: transplantation. J Heart Lung Transplant 14:163, 1995
8. Feucht H: Complement C4d in graft capillaries—the missing link in the recognition of humoral alloreactivity. Am J Transplant 3:646, 2003
9. Salmon J: Abnormalities in immune complex clearance and Fcγ receptor function. In: Wallace DJ, Hahn BH, (eds): Dubois' Lupus Erythematosus. Baltimore: Lippincott Williams & Wilkins 2002, p 221
10. Schur PH, Klickstein L: Complement and systemic lupus erythematosus. In: Wallace DJ, Hahn BH, (eds): Dubois' Lupus Erythematosus. Baltimore: Lippincott Williams & Wilkins 2002, p 245

11. Manzi S, Navratil JS, Ruffing MJ, et al: Measurement of erythrocyte C4d and complement receptor 1 in systemic lupus erythematosus. Arthritis Rheum 11:3596, 2004

12. Chen CH, Klickstein LB, Lai JH, et al: Evaluate erythrocyte C4d and complement receptor 1 ration in systemic lupus erythematosus by using CR1-2B11. APLAR J Rheum 9(suppl 1): A14, 2006

13. Manzi S, Ahearn JM, Salmon J: New insights into complement: a mediator of injury and marker of disease activity in systemic lupus erythematosus. Lupus 413:298, 2004

14. Wei J, Chang CY, Chuang YC, et al: Successful heart transplantation after 13 hours of donor heart ischemia with the use of HTK Solution: a case report. Transplant Proc 37:2253, 2005

15. Beletskaya LV, Kupriyanova AG, Kormer AY, et al: Rheumatoid course of human (vascular) rejection after heart allotransplantation. Bull Exp Biol Med 142:363, 2006

16. Tan CD, Baldwin WM, Rodriguez ER: Update on cardiac transplantation pathology. Arch Pathol Lab Med 131:1169, 2007

17. Crespo MG, Veiga A, Domenech N, et al: Humoral heart rejection (severe allograft dysfunction with no signs of cellular rejection or ischemia): incidence, management, and the value of C4d for diagnosis. Am J Transplant 5:2560, 2005

18. Abrams J, Amir O, Etheridge WB, et al: Histological findings proving the existence of humoral rejection in a cardiac allograft. Cardiovasc Pathol 16:38, 2007

19. Vallhonrat H, Williams WW, William G, et al: Complement activation products in plasma after heart transplantation in humans. Transplantation 71:1308, 2001

20. Chen CH, Ghiran I, Beurskens FJM, et al: Antibody CR1-2B11 recognizes a non-polymorphic epitope of human CR1 (CD35). Clin Exp Immunol 148:546, 2007