Int J Clin Exp Pathol 2017;10(4):4772-4779
www.ijcep.com /ISSN:1936-2625/IJCEP0049109

Original Article

Acute cellular rejection and antibody-mediated rejection in endomyocardial biopsy after heart transplantation: a retrospective study from a single medical center

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Received January 18, 2017; Accepted February 22, 2017; Epub April 1, 2017; Published April 15, 2017

Abstract: Aims: Endomyocardial biopsy (EMB) is the routine procedure for monitoring the outcome of heart grafts. This article reports acute cellular rejection and antibody-mediated rejection after heart transplantation, as detected by EMB. Methods: We retrospectively evaluated 496 patients who underwent heart transplantation in Fuwai Hospital between August 2004 and June 2015. The EMB specimens were used for evaluating acute cellular rejection (ACR) and antibody-mediated rejection (AMR) by histopathology and immunohistochemistry. Results: Of 496 patients who underwent HTX, a total of 856 EMBs were performed in 261 patients. Among 850 myocardium biopsies, 425 (52.9%, 425/850) showed no evidence of ACR and AMR. ACR was seen in 345 EMBs (40.6%, 345/850) with mild rejection observed (grade 1R) in 318 cases (37.4%, 318/850), moderate rejection in 25 cases (2.9%, 25/850) EMBs and severe rejection (3R) in 2 cases (0.23%, 2/850). Antibody-mediated rejection (AMR) was found in 8 EMBs, including 5 AMR alone and 3 AMR mixed with ACR. CD68 positive macrophages were found in all 8 EMBs with AMR and C4d staining was positive in 6 cases (75.0%). S6RP and pS6K were positive in 7 (87.5%) and 5 (62.5%) EMBs with AMR separately. Conclusions: Intravascular macrophages are a sign of microvascular inflammation and the earliest evidence of antibody-mediated allograft injury. Phosphorylated S6RP has the highest sensitivity in diagnosis of AMR, followed by C4d and pS6K. C4d is still a useful marker for AMR diagnosis.

Keywords: Endomyocardial biopsy, cardiac transplantation, cellular rejection, antibody-mediated rejection

Introduction

Heart transplantation (HTX) is an established treatment for terminal heart failure [1, 2]. In 2010, a total of 3,892 heart transplants were reported to the International Society for Heart and Lung Transplantation (ISHLT) Registry [3]. Therefore, in managing affected patients, the close monitoring of lesions after HTX and during rejection, is a major challenge for cardiologists and heart surgeons. Many methods have been performed for rejection monitoring, and noninvasive methods such as cardiac echocardiography or MRI have been demonstrated not to retain the sensitivity and specificity [4]. Some serum immune circulating molecules such as anti-HLA antibodies, anticardiac myosin or anti-endothelial cell antigens, as well as plasma complement fragments C4d or C56-9, were proposed as biomarkers of graft rejection. However, no clear association between these molecules and heart rejection was confirmed [5]. Until now, endomyocardial biopsy (EMB) is still the most widely used routine procedure for monitoring the heart graft [6].

Acute cellular rejection (ACR) occurs most frequently and is characterized by the presence of inflammatory cells in the myocardium. It is classified into four grades: 0R, 1R, 2R and 3R [7]. Both the 2R and 3R grades require additional immunosuppressive treatment. ACR has been described precisely and the advent of immunosuppressants significantly lowered its frequency.

Antibody-mediated rejection (AMR) is also drawing attention because of its role in cardiac allograft rejection and graft loss [8-10]. AMR is defined as allograft rejection caused by anti-

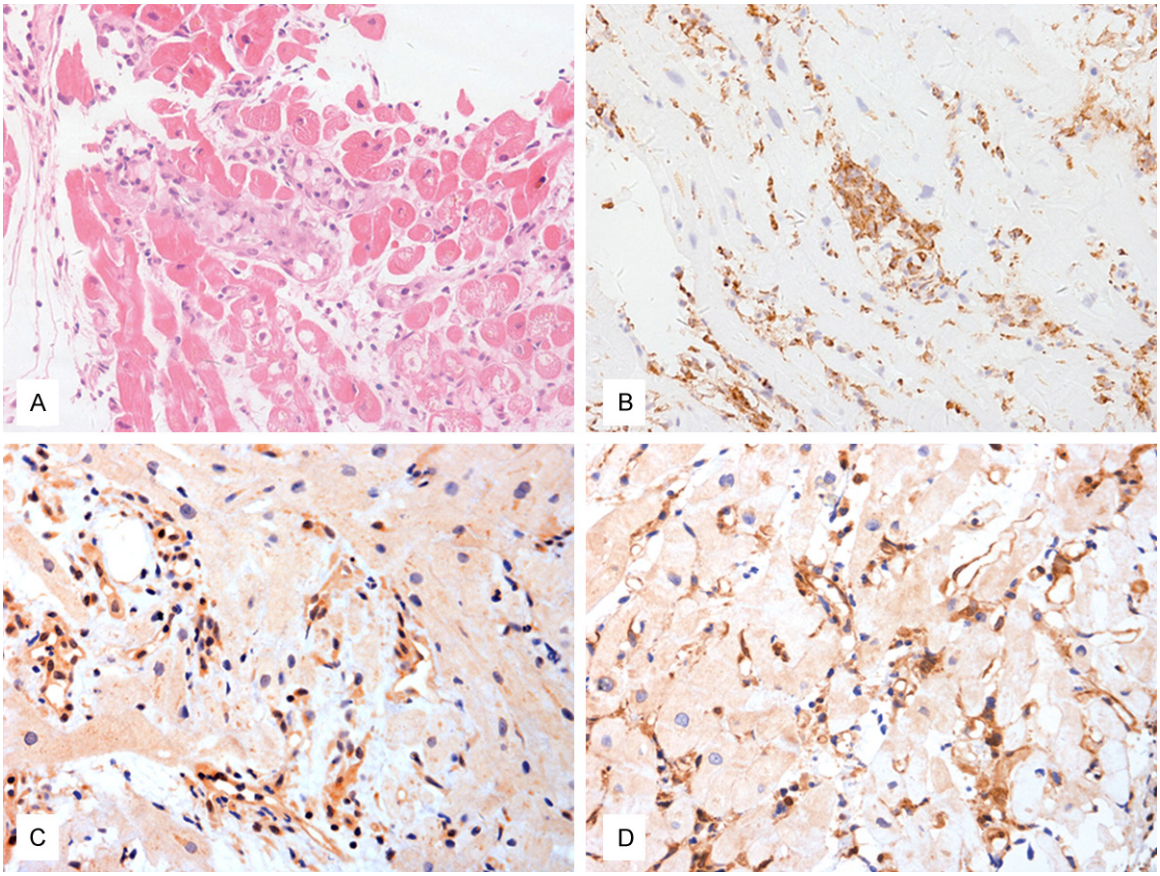

Figure 1. Morphologic and immunologic features of antibody-mediated rejection. A: Activated mononuclear cells fill and expand interstitial vessels (arrow shows). B-D: Immunologic features of AMR, B: CD68-positive macrophages fill vascular lumens. C: p-S6K strong and diffuse staining of capillaries. D: p-SRP strong and diffuse staining of capillaries. Original magnification, 200×.

bodies directed against donor-specific human leukocyte antigen (HLA) molecules, blood group antigen (ABO)-isoagglutinins, or endothelial cell antigens [11]. AMR occurs in allosensitized individuals exposed to transfusion, gestations, transplants, or circulatory assistance devices [12]. The incidence of acute AMR may be as high as 15% during the first post-transplant year and confers a high risk for the later development of transplant coronary artery disease (TCAD) [12]. In 2011, based on an updated consensus, the AMR diagnosis was switched from a clinical to a pathological condition [13]. The diagnosis of AMR from biopsies was facilitated by the recognition of diffuse deposition of C4d, a breakdown product of the complement component C4, in capillaries [14]. In some c4d-negative AMR, the downstream effectors of the mammalian target of rapamycin (mTOR), phosphorylated 70S6 kinase (S6K) and S6 ribosomal protein (S6RP), were detected in monitoring the proliferation of endothelia [15].

The present study reports EMB findings in patients who underwent heart transplantation between August 2004 and June 2015 at the Fuwai hospital. The study also investigates whether the signal transduction pathways involved in AMR have the potential to improve the diagnosis of cardiac AMR and guide the development of new therapeutic strategies for AMR.

Materials and methods

EMB and patients

We analyzed all consecutive EMBs from patients who underwent an HTX at the Center for Heart Transplantation, between August 2004 and June 2015. A total of 496 patients underwent HTX during this 11-year study period, of which 261 patients underwent endomyocardial biopsies (EMB). EMBs at the interventricular septum on the side of the right ventricle were

obtained either as routine surveillance protocol biopsies or as a diagnostic tool in cases with allograft dysfunction and clinically suspected rejection. The regular biopsy schedule was as follows: every two weeks in the first month, every three months in the first years, and then every 6 months for the next 3 years.

The patients' clinical data including age, sex, primary heart disease and survival time after heart transplantation collected during the follow-up were reviewed. Cardiac allograft dysfunction after HTX was defined as left ventricular ejection fraction <50%, as measured by echocardiography, in the presence of signs and symptoms of heart failure such as cardiogenic shock, hypotension, decreased cardiac index and/or a rise in capillary wedge or pulmonary pressures [12]. The hospital ethics committee approved the study protocol.

Diagnosis of rejection

The biopsy specimens (usually 5 biopsy fragments) were fixed in 10% formalin and processed using a fast embedding program that yielded initial results within 12 hours. Sections were cut for hematoxylin-eosin (H&E) and Masson's Trichrome staining. Histological evaluation was performed by two pathologists in accordance with the ISHLT 2004 Working Formulation for Biopsy Diagnosis of Cardiac Allograft Rejection [7] and the ISHLT 2013 Working Formulation for the standardization of nomenclature in the pathologic diagnosis of antibody-mediated rejection in heart transplantation [13]. Accordingly, "pAMR" was refined in 4 subcategories: pAMR0 when no histopathologic and immunopathologic feature of AMR is seen on EMB; pAMR1, or "suspicious AMR", when only histopathologic [pAMR1(H+)] or immunopathologic [(pAMR1(I+)] features are present; pAMR2 when both histopathologic and immunopathologic features occur simultaneously; and pAMR3, or "severe AMR", when additional signs of hemorrhage, intense inflammation, and endothelial cell pyknosis are evident [13].

C4d and CD68 immunostainings were performed in patients with histological or clinical suspicion of antibody-mediated rejection. Immunostainings for C4d and CD68 were performed using affinity-purified anti-human C4d and CD68 rabbit polyclonal antibodies (Biomedica Gruppe, Vienna). We considered C4d positivity in the setting of multifocal or diffuse (>50%) capillaries involvement and CD68 positivity when intravascular CD68-positive macrophages are present in >10% of capillaries (**Figure 1**) according to the new ISHLT classification [13].

Immunohistochemical analysis and grading of the phosphorylated S6 kinase and phosphorylated 70 S6-kinase protein

Phosphorylated S6 kinase (S6RP) and 70 S6-kinase (S6K) protein immunostainings were performed in patients with histological or clinical suspicion of antibody-mediated rejection. The patients with only acute cellular rejection or no rejection were also selected for negative control. Immunohistochemical staining was performed as described elsewhere using antibodies against phospho-S6RP (Ser240/244, Catalog No. 14236) and phospho-70S6-kinase protein (Thr421/Ser424, Catalog No. 9204), all purchased from Cell Signaling Technology Inc. (MA, USA). Briefly, endomyocardial biopsy sections were deparaffinized and rehydrated. Antigen was recovered in 10 mmol/L sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was inhibited using 3% hydrogen peroxide in methanol for 15 minutes. The sections were then blocked with 10% normal goat serum in phosphate-buffered saline (PBS). Sections were incubated with 100 μl diluted primary antibodies (1:50 for p-S6RP and p-70S6K) overnight at 4°C, followed by incubation with the IHC Detection Reagent (HRP, Rabbit. Cell Signaling Technology, Inc, CA) for 30 minutes at room temperature. The sections were then developed with a diaminobenzidine kit (DAB kit; Thermo Fisher Scientific Inc. MA, USA), and counter-stained with weak hematoxylin. Cardiac biopsies were scored by two study-blind cardiac pathologists. Positive EC staining results for phosphorylated forms of S6RP and SP70 was scored as follows: Grade 0 = no staining; Grade 1 = rare staining of single cells; Grade 2 = focal staining, several positive capillaries, but only in one region of the biopsy specimen and involving less than one third of the biopsy; and Grade 3 = multifocal to diffuse staining. A score ≥2 was considered positive [15].

Statistical analysis

Continuous variables were expressed as means and standard deviations and categorical variables as counts and percentages. Sensitivity

Table 1. Patient characteristics

Variables	No. (%) or Mean ± SEM (n=261)
Age in years	43.7±13.9
Male	211 (80.8)
Primary heart disease	
Non-ischemic cardiomyopathy	199 (76.2)
Ischemic cardiomyopathy	46 (17.6)
Valvular heart disease	9 (3.4)
Primary heart tumor	2 (0.8)
Secondary heart disease*	5 (1.9)
Retransplantation	2 (0.8)
Time since transplant	
<2 years	34 (13.0)
2-5 years	47 (18.0)
≥6 years	180 (69.0)

*Including: Duchenne myodystrophia, Behcet's aortitis and Marfan's syndrome.

and specificity of Phosphorylated S6 kinase and 70 S6-kinase were determined by the Kappa test to compare categorical variables. The actual *P* values were calculated for small frequencies. $P<0.05$ was considered significant. Statistical analyses were performed using the SPSS16.0 for Windows software (IBM Corporation; Armonk, NY).

Results

Acute cellular rejection

In 261 patients, with an average age of 43.7±13.9 years (range, 13-66 yrs), there were altogether 856 EMB events. The characteristics of these 261 patients included in the study are given in **Table 1**. Of 856 biopsies, six cases in which the harvested EMB fragments contained no myocardial tissue were excluded from the study. Among remaining 850 biopsies, 425 (52.9%, 425/850) showed no evidence of cellular rejection (scored grade 0R) or antibody-mediated rejection. Acute cellular rejection was seen in 345 EMB (40.6%, 345/850) cases, including mild rejection (grade 1R) in 318 EMB (37.4%, 318/850) cases, moderate rejection in 25 (2.9%, 25/850) EMB cases and severe rejection (3R) in 2 (0.23%, 2/850) EMB cases.

Antibody-mediated rejection and immunological features

Of 850 EMBs, pathologic AMR (pAMR) were observed in 8 EMBs including 5 AMR alone and 3 AMR accompanied by ACR. Typical histopathological features, such as swollen endothelial cells and intravascular macrophages, could be identified in all 8 EMBs, while interstitial edema was observed in three EMBs (**Figure 1**). The histopathological and immunological features of 8 EMBs with AMR are shown in **Table 2**. Diffuse C4d staining (>50%) was seen in 6 EMBs which were labeled C4d (+) AMR and focal C4d staining (<10%) in two which were labeled C4d (-) AMR. Diffuse CD68 staining (>10%) was seen in all 8 EMBs.

To assess the cell-proliferation pathways activated *in vivo* by the HLA Class I molecules in the capillary endothelium, the expression of p-S6K and p-S6RP proteins was examined by immunohistochemistry on all 8 pAMR 2 EMBs. Eight patients with clinical suspicion of AMR but no histological feature (pAMR0) were also examined the expression of p-S6K and p-S6RP proteins. **Figure 2** illustrates the staining pattern of positive p-S6K and p-S6RP in EMBs. The association between p-S6K and p-S6RP of capillary ECs and AMR, with or without C4d, is shown in **Table 3**. Seven of eight patients (87.5%, patient No. 1, 2, 4-8) with AMR demonstrated multifocal to diffuse capillary staining for p-S6RP, whereas only one patient (12.5%, patient No. 3) demonstrated focal staining (grade 1). Five of eight patients (62.5%, No. 1, 2, 4, 7, 8) with AMR showed multifocal to diffuse capillary staining of p-S6K, while three (37.5%, No. 3, 5, 6) exhibited focal staining (grade 1). All 8 patients with pAMR0 were all negative immunity for p-S6K and p-S6RP with the specificity of 100% (**Table 3**).

Time schedule of lesions

Accordant to our biopsy time schedule, the frequency of EMB was the highest (231 EMB) in the first month, followed by EMBs performed in months 3 to 6 (156 EMB). The frequency of 1R rejection was higher during months 1-6 than during months 6-12 and >1 year period (41.7% vs 28.0% and 38.1%, P=0.033). The most grade 2R/3R rejections occurred during months 1-6 (n=17, 60.7%). Antibody-mediated rejection was seen in eight cases (5 AMR alone and 3 AMR accompanied with other lesions) and occurred in the first month (6) and months 1-3 as seen in **Figure 2**.

Discussion

This article represents a large, 11-year retrospective study of heart transplant cases from

Table 2. Histopathological and immunological features of seven biopsies with AMR

No	Sex	Age	Time after HTX	Syndrome and signs	Pathological diagnosis	Histological features	Immunopathological features			
							C4d (+)	CD68 (+)	p-S6R (positive score)	p-S6K (positive score)
1	M	47 y	6 d	Hypotension LVEF43%	ACR1R+AMR1	EC swollen, MC accumulation	>50%	>50%	3	3
2	M	35 y	5 d	Fatigue, LVEF 45%	AMR1	EC swollen, MC accumulation, interstitial edema	<10%	>50%	3	3
3	M	14 y	14 d	IVS thicken	ACR1R+AMR1	EC swollen, MC accumulation	<10%	>50%	1	1
4	M	48 y	48 d	Tachyarrthymia Hypotension	AMR1	EC swollen, MC accumulation interstitial edema	>50%	>50%	3	3
5	F	53 y	1 d	NT-proBNP increase	AMR1	EC swollen, MC accumulation	>50%	10-50%	3	1
6	M	45 y	12 d	Hypotension	AMR1	EC swollen, MC accumulation	>50%	>50%	2	2
7	F	53 y	8 d	Low cardiac output and hypotension	AMR1	EC swollen, MC accumulation	>50%	>50%	3	1
8	M	34 y	40 d	BNP increase and Tachyarrthymia	AMR1+ACR1R	EC swollen, MC accumulation interstitial edema	>50%	>50%	3	3

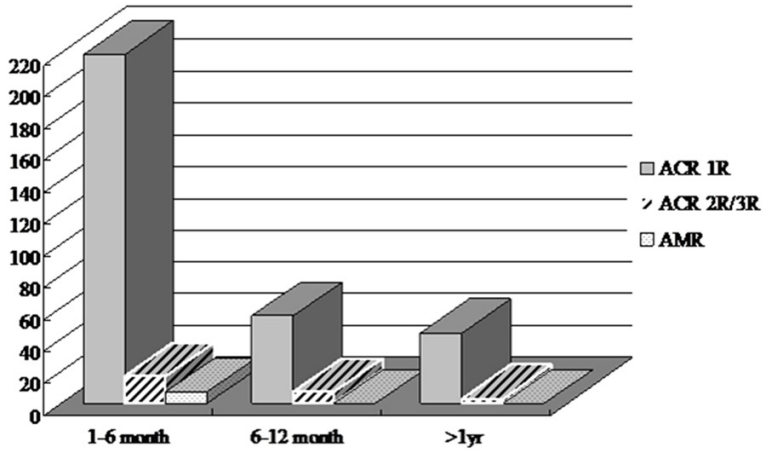

Figure 2. Time schedule of ACR and AMR after HTX.

Table 3. Associations among capillary phosphorylation S6K, phosphorylation S6RP and classification of pAMR

Phosphorylated staining	Immunity	pAMR0	pAMR2	*P* value
S6K	positive	0	5	
	negative	8	3	0.007
S6RP	positive	0	7	
	negative	8	1	0.000

the largest cardiovascular referral hospital in China, in which 850 right ventricle EMBs were analyzed. A lower incidence of moderate and severe acute cellular rejection (2.9% and 0.3%) was observed in this study in comparison to other studies (7.2%-26.6% and 1.3%) [16, 17]. The incidence of mild rejection (Grade 1R) in our study (40.6%) was similar to that observed in the other centers (45.1%) [16]. In a series of 1896 EMBs in Germany [16], mild (grade 1R), moderate (grade 2R) and severe (grade 3R) rejections were seen in 45.1%, 7.2% and 1.3% of EMBs, respectively. The low incidence of moderate and severe cellular rejection seen in the present study point to good compliance and effective anti-rejection therapy after transplantation. We also found that most 1R and 2R ACR cases occurred during the first 6 months. The time schedule of ACR was similar as in the other reports. In Strecker's report, the majority of 2R and 3R occurred within the first month [15].

Antibody-mediated rejection was rare in our study, with only 0.94% of 850 EMB. This prevalence is slightly lower than reported in other studies (from 1.4- to 3%) [14, 17, 18]. This disparity can also be explained, in part, by the varying presence of risk factors in patient populations. AMR risk factors include female multiparity, a history of blood transfusion, prior transplantation, the use of left ventricular assist device, congenital heart disease and cytomegalovirus seropositivity, etc [8, 12, 19]. In the present study, these risk factors were not found, except in two patients with a history of prior heart transplantation (**Table 1**).

In the present study, positive CD68 findings were observed in all EMBs, with a pathological diagnosis of AMR, including seven symptomatic and one asymptomatic patient. This result is in accordance with the previous observation of Fedrigo et al [19], who used CD68 immunostaining to evaluate the role of intravascular macrophages in the diagnosis of early and late AMR. In their study, in the early period, intravascular macrophages were more common in symptomatic (3 of 3, 100%) than asymptomatic (3 of 11, 27.3%) patients [19]. In the present study, one EMB had the obvious histological features of AMR, but the only immunohistochemical feature was the CD68 positive status. Therefore, it has been suggested that intravascular macrophages are a sign of microvascular inflammation and represent the earliest evidence of antibody-mediated allograft injury. If recognized early, before the complement deposition on tissue, it could represent a potential therapeutic target for prevention of consequent graft dysfunction and failure [19].

This study showed that pS6RP has the highest sensitivity in diagnosis of AMR, followed by C4d and pS6K (87.5%, 75.0% and 62.5%, respectively). This result is in accordance with the previous study by Lepin et al [20], but distinct from Li et al [15] and Tible et al [21]. In Lepin's study, nineteen of twenty (95%) patients with AMR showed multifocal or diffuse capillary staining for pS6RP. However, in Li's study, it seemed that pS6k has higher sensitivity compared to

pS6RP (53.1% vs 36.7%) in AMR. Tible and his colleagues also showed that pS6k and pS6RP are found with a higher frequency in pAMR2 EMB specimens (77.8% and 55.6%, respectively) than in pAMR0 (3.3% and 3.3%, respectively) and pAMR1 (47.6% and 14.3%, respectively) [21]. This discrepancy may be partly ascribed to the differences in the number of patients. In our series, only eight patients had the histological features of AMR. In Li and Tible's studies, there were relatively large quantities of EMBs with AMR (49 and 37, respectively) [15, 21].

Recently, the sensitivity of C4d in diagnosis of AMR was questioned in renal [22] and cardiac AMR [21]. In the study of Tible and colleagues, C4d positive was found in only 22.2% of pathological AMR in cardiac AMR [21]. Unlike his report, the incidence of C4d positive was relatively high with 75.0% of AMR in our series. This result was in agreement with another study, in which Crespo-Leiro and his colleagues found that all six patients with clinical AMR were C4d positive [17]. Together with their study, our results strongly suggest that C4d is still a useful marker of AMR.

Limitations

Two limitations of the present study are the relatively small sample size and the absence of routine DSA testing in AMR patients. So the correlation of pS6RP and pS6K with DSA could not be evaluated in the present study. Further work and observation are warranted.

Conclusions

The incidence and severity of acute cellular rejection episodes were lower among the patients in our center in comparison to similar earlier reports. The incidence of antibody-mediated rejection was also very low. However, cardiologist and pathologist must still be aware of its potential role in graft dysfunction in AMR patients. Except for C4d and CD68, additional endothelium injury transcripts, such as pS6RP and pS6K, should be used to identify AMR. To the best of our knowledge, this is the first study reporting EMBs pathology results in China from a single institution.

Acknowledgements

This study was supported by grants from National Natural Science Foundation of China (81341045) and Peking Union Medical College Youth Fund (33320140038), Republic of People's Republic of China.

Disclosure of conflict of interest

None.

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