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Phosphorylated S6 Kinase and S6 Ribosomal Protein are Diagnostic Markers of Antibody Mediated Rejection in Heart Allografts

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Abstract

Background—Anti-MHC class I alloantibodies have been implicated in the processes of acute and chronic rejection. These antibodies (Ab) bind to endothelial cells (EC) and transduce signals leading to the activation of cell survival and proliferation pathways, including Src, FAK, mTOR, and downstream targets ERK, S6 kinase (S6K) and S6 ribosomal protein (S6RP). We tested the hypothesis that phosphorylation of S6K, S6RP and ERK in capillary endothelium may serve as an adjunct diagnostic tool for antibody mediated rejection (AMR) in heart allografts.

Methods—Diagnosis of AMR was based on histology or immunoperoxidase staining of paraffin-embedded tissue consistent with 2013 ISHLT criteria. Diagnosis of acute cellular rejection (ACR) was based on ISHLT criteria. Endomyocardial biopsies from 67 heart transplant recipients diagnosed with acute rejection [33 with pAMR, 18 with ACR (15 with grade 1R, 3 with grade >2R), 16 with pAMR+ACR (13 with 1R and 3 with >2R)] and 40 age- and gender-matched recipients without rejection were tested for the presence of phosphorylated forms of ERK, S6RP and S6K by immunohistochemistry.

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Results—Immunostaining of endomyocardial biopsies with evidence of pAMR showed significant increase in expression of p-S6K and p-S6RP in capillary EC compared to controls. A weaker association was observed between pAMR and p-ERK.

Conclusions—Biopsies diagnosed with pAMR often showed phosphorylation of S6K and S6RP, indicating that staining for p-S6K and p-S6RP is useful for the diagnosis of AMR. Our findings support a role for antibody-mediated HLA signaling in the process of graft injury.

Keywords

endomyocardial biopsies; cardiac allograft; s6 kinase; antibody-mediated rejection; C4d

Introduction

Antibody-mediated rejection (AMR) is emerging as a leading cause of cardiac and renal [1-4] allograft rejection and graft loss. Heart transplant recipients can present with AMR anytime postoperatively, ranging from a few days to years. AMR is predominantly mediated by alloantibodies to donor human leukocyte antigens (HLA), and is characterized by the deposition of complement and immunoglobulin within the graft and the presence of circulating donor-specific HLA antibodies in the recipients [1, 5, 6]. The incidence of acute AMR may be as high as 15% during the first post-transplant year and confers high risk for the later development of transplant coronary artery disease (TCAD) [7]. TCAD constitutes a severe and irreversible complication of heart transplantation and is a major barrier to long-term success of cardiac transplantation [8-10]. Therefore, interruption of the AMR disease process may protect patients from TCAD.

Because of technical advances in the ability to detect alloantibodies in the circulation and in the graft, the contribution of anti-HLA antibodies to human allograft rejection has been increasingly recognized. Deposition of C4d, a cleavage product of complement, in capillaries was shown to be a useful marker of AMR in renal, as well as cardiac allografts and strongly correlated with the presence of donor specific antibodies (DSA) [11-13]. Nevertheless, the sensitivity of capillary C4d staining in cardiac biopsy specimens remains controversial [14]. For instance, negative staining for C4d occurs occasionally during the course of AMR [15] and positive staining occurs in the absence of symptomatic AMR [13, 16]. Capillary deposition of C4d in DSA-negative recipients raises the possibility of antibody-independent complement activation. In addition, the morphological classification of pAMR has limited sensitivity and reproducibility as discussed in the 2005 ISHLT Consensus Working Formulation [17]. Therefore, discovery of new molecular diagnostic markers of AMR promises to improve diagnosis and management of cardiac allograft rejection.

The production of Ab to donor HLA antigens before or after cardiac transplantation is a major risk factor for the development of AMR [5, 6]. The pathological effect of DSA binding to the transplanted organ is likely to involve signaling pathways elicited by ligation of class I and class II molecules on the surface of endothelial cells (EC) and smooth muscle cells [18-20]. Engagement of HLA class I molecules by anti-HLA Ab increased the activation of Extracellular-signal-regulated kinases (ERK1/2) [21, 22], p70 S6 Kinase (S6K)

and S6 ribosomal protein (S6RP) through the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) and 2 (mTORC2) and stimulated mTOR-dependent cell proliferation in EC and smooth muscle cells [21, 23-25]. Ligation of HLA class II molecules on cultured EC also stimulated an increase in phosphorylation of S6RP [26]. We hypothesized that the activation of EC might manifest in allografts of patients with donor specific class I and class II Ab. Indeed, S6RP was highly activated in biopsies with AMR in a previous study [26]. We observed that increased capillary staining for p-S6RP and p-S6K in cardiac biopsies was highly associated with AMR. Interestingly, p-S6K staining was increased in patients diagnosed with both C4d+ and C4d- AMR, suggesting that the phosphorylation of S6K is a superior indicator of AMR and CAV compared with C4d. Elucidation of the signal transduction pathways involved in AMR has the potential to improve the diagnosis of cardiac AMR and to guide the development for the new therapeutic strategies for AMR and consequent TCAD.

Material and Methods

Patient study population

Informed consent was obtained from all patients and the study was approved by the UCLA Institutional Review Board. The patient population consists of 107 heart allograft recipients transplanted between January 1995 and December 2005, which was unique from our previous report [26]. Endomyocardial biopsy specimens were obtained from the right ventricle. Surveillance protocol biopsies were obtained weekly in the first month, biweekly in the 2nd month, then once in months 3, 4, 5, 6, 9, 12, 18 and 24 post-transplant. Additional biopsies were performed when there was clinical suspicion of rejection and 7–10 days after treatment of documented rejection in order to confirm resolution of rejection. Cardiac tissue was placed immediately into Bayley's fixative and embedded in paraffin. Paraffin blocks were serially cut into 3- μ m-thick sections [27]. Characteristics of the patient population are listed in Table 1.

Diagnosis of Rejection

AMR diagnosis was based on histological and immunohistochemical criteria (i.e., pAMR) irrespective of serology consistent with the 2013 ISHLT Working Formulation [28], and diagnostic criteria for ACR as in [17, 29]. pAMR criteria comprise a combination of histological and immunohistochemical features involving myocardial capillaries, which show evidence of endothelial injury with swelling of cytoplasm and nuclear enlargement. Immunohistochemical features include CD68 and/or C4d staining, with prominent linear accumulation of intravascular macrophages sometimes associated with interstitial edema and patchy interstitial hemorrhage. Immunoperoxidase staining was performed to document C4d deposition and CD68 to identify macrophages in capillaries, in combination with a vascular marker, CD31 or CD34.

Immunohistochemical analysis of phosphorylated S6 kinase and S6 ribosomal protein and grading

Immunohistochemical staining was performed as previously described [26] by using antibodies against phospho-S6K (Thr421/Ser424) (Cat# 9204), phospho-S6RP (Ser

235/236) (Cat# 2211), and phosphop44/42 ERK 1/2 (Thr202/Tyr204) (clone 20G11, Cat# 4376) purchased from Cell Signaling Technology. The specificity of these antibodies was verified by Western blotting and immunohistochemical analysis [21, 23, 25, 26, 30-32]. Briefly, endomyocardial biopsy sections were de-paraffinized and rehydrated. Antigen was recovered in a steamer with EDTA buffer (PH 8.0) for phospho-Akt and 10 mM sodium citrate buffer (pH 6.0) for C4d, phospho-ERK (p-ERK), phospho-S6K (p-S 6K) and phospho-S6RP (p-S6RP). Endogenous peroxidase activity was inhibited by 3% hydrogen peroxide in methanol for 15 min. Sections were then blocked with 10% normal goat serum (NGS) in PBS. Sections were incubated with 100 µl diluted primary antibodies (1:50 for p-ERK; 1:20 for p-S6K; 1:50 for p-S6RP) for overnight at 4°C, 1:200 dilution of biotinylated goat anti-rabbit IgG (Vector Labs, Burlingame, CA) for 40 min at room temperature, and 1:1000 dilution of horseradish peroxidase avidin D (HRP, Vector), developed with DAB kit (Vector), and then counterstained with weak hematoxylin. Cardiac biopsies were scored by two blinded cardiac pathologists. Positive EC staining for phosphorylated forms of S6K, S6RP, and Erk was scored as follows: grade 0 = no staining, grade 1 = rare staining of single cells; grade 2 = focal staining, several positive capillaries, but in only one region of the biopsy involving less than 1/3 of the biopsy; and grade 3+ = multifocal to diffuse staining. A score of 2 or greater was considered positive.

Evaluation of Anti-HLA Antibodies

Sera samples were analyzed for antibodies directed against HLA class I (A, B, C) and class II (DR, DQ, and DP) antigens using the Gen-Probe Luminex PRA and antibody specificity reagents (San Diego, CA). In brief, 5 µl of HLA class I or II antigen-coated Luminex beads were incubated with 20 µl of patient's serum sample for 30 min. Unbound excess serum was removed by washing and the microparticles were stained with 100 µl phycoerythrin conjugated goat antihuman immunoglobulin (Ig) G antibody for 30 min. Particle fluorescence was assessed by Luminex 100 IS (Luminex, Austin, TX). Additional Single Antigen Antibody Identification Assays (One Lambda) were run on positive sera to confirm the antibody specificity assignment. Antibodies were considered positive if the median fluorescence intensity was more than 1000.

Statistical analysis

Patient characteristics among the four groups (C4d-/pAMR-, C4d+/pAMR-, C4d-/pAMR+, and C4d+/pAMR+) were summarized using means $\pm$ standard deviations for continuous variables and counts (percents) for categorical data. Associations between groups and phosphorylation scores were tested utilizing Fisher's exact tests in single variable analyses and were assessed via logistic regression in multivariable analysis. Regarding logistic regression, the Hosmer-Lemeshow test was used to judge model fit and results did not indicate lack of model fit (χ^2 (8) = 10.54, P=0.23). All p-values were two-sided and p<0.05 was considered significant.

Results

Based on the histopathologic and immunohistochemical diagnosis of their cardiac biopsies, the study population was categorized into four groups: C4d-/pAMR-, C4d+/pAMR-, C4d-/

pAMR+, and C4d+/pAMR+. Using propensity score analysis, 30 subjects each from C4d-/pAMR-, C4d+/pAMR-, and C4d+/pAMR+ and 22 subjects from C4d-/pAMR+ were initially selected and matched for age, gender, and time to biopsy. However, due to limited cardiac tissue for analysis, one patient from C4d-/pAMR-, one from C4d+/pAMR-, and three from C4d+/pAMR+ had to be excluded from this study. Therefore, immunohistochemical stains for CD68 and C4d were performed on endomyocardial biopsies of 107 cardiac allograft recipients. The characteristics of the study population are presented in Table 1. Thirty-three patients were diagnosed with pAMR only; 15 were diagnosed with ACR 1R, 3 with ACR >2R; 13 were diagnosed with both pAMR and ACR 1R, 3 with pAMR + ACR >2R, and 40 patients had no evidence of rejection.

To assess the cell proliferative pathways activated in vivo by HLA class I molecules in capillary endothelium, protein expression of p-S6K, p-S6RP, and p-ERK were examined by immunohistochemistry. Figure 1 illustrates the staining pattern of positive p-S6K, p-S6RP and p-ERK in representative cardiac transplant samples. The distribution of p-S6K was predominantly nuclear, and that of p-S6RP was cytoplasmic; while the distribution of p-ERK was both cytoplasmic and nuclear. Biopsies with no (grade 0) or rare (grade 1) EC staining were considered negative. Focal (grade 2) or multifocal to diffuse (grade 3+) EC staining were considered positive. Background staining of the intercalated discs for p-S6RP was consistently seen in a majority of the biopsies. Strong staining of necrotic myocytes and necrotic fat cells by p-S6RP and p-ERK was also observed in biopsies with myocardial damage. Positive staining of interstitial and intravascular leukocytes for p-S6K and p-S6RP was also seen in some biopsies.

The association between p-S6K, p-S6RP, and p-ERK staining of capillary ECs and pAMR with or without C4d positivity was analyzed (Table 2). A strong association between pAMR and expression of p-S6K and p-S6RP in capillary EC was observed ($p < 0.001$). Twenty-six of 49 patients (53.1%) with pAMR demonstrated focal or multifocal to diffuse capillary staining for p-S6K, while only 5 of 58 patients (8.6%) without pAMR demonstrated grade 2 or grade 3+ capillary staining for p-S6K. Of these five patients, three had no histopathologic evidence of ACR, two had ACR 1R. Similarly, 18 of 49 patients (36.7%) with pAMR showed focal or multifocal to diffuse capillary staining of p-S6RP, while only 5 out of 58 patients (8.6%) without pAMR exhibited grade 2 capillary staining for p-S6RP. The association between ERK phosphorylation, pAMR and C4d staining was more complicated. Analyses revealed a borderline significance which was likely due to a single group (C4d+/pAMR-) wherein ERK phosphorylation was different; however, the distribution of ERK phosphorylation was the same among the other three classifications.

To further determine the association between the staining grades and 95% confidence interval of S6K, S6RP and ERK and the classification of AMR+, we calculated the odds ratio of different phosphorylation grades in pAMR+ or pAMR- patient classifications (Table 3). The odds ratio for AMR risk was 52 and 49 for S6K staining grade at 2 and 3+, respectively. Phosphorylated S6RP yielded an odds ratio of 4 and 10 for grades 1 and 2, 3+, respectively. We found that increasing levels of S6K phosphorylation exhibited strong associations with the diagnosis of pAMR, and S6RP phosphorylation score 2 or greater significantly increased the risk of AMR. However, when S6K and S6RP phosphorylation

levels were taken into account, ERK phosphorylation did not further increase the risk of AMR (Table 3), suggesting that p-S6K and p-S6RP are sufficient to predict AMR.

The association between ACR and phosphorylation of S6K, S6RP and ERK in presence or absence of pAMR was analyzed (Table 4). By stratifying ACR as a confounder, the subset of biopsies with “pure” pAMR remained associated with capillary phosphorylation staining when compared with biopsies without rejection. In contrast, within the pAMR negative group, no grade of ACR was significantly associated with phosphorylation of any markers. In the pAMR+ group, the concurrence of ACR slightly reduced the association of pAMR with phosphorylated S6K ($p=0.01$, Table 4). Caution should be exercised in interpretation of this data as patients were selected retrospectively for AMR and the study was not designed to address the relationship between ACR and phosphorylated signaling proteins.

Given that phosphorylation of S6K in capillaries was a superior marker of pAMR diagnosis compared with S6RP, the capacity of capillary phosphorylated S6K to predict AMR in C4d positive and negative groups was determined. We found that in patients diagnosed with pAMR, allograft biopsies had a higher probability of capillary p-S6K staining irrespective of C4d positivity (Figure 2A). In addition, only the C4d+pAMR group had a high incidence (67%) of DSA, while the C4d-pAMR group had lower proportion of DSA (23%) (Figure 2B). These results suggest that phosphorylation of S6K is sensitive for detection of both C4d + and C4d- pAMR, as well as pAMR occurring with no detectable DSA.

Discussion

We report herein that activation of intracellular signaling molecules in heart allograft endothelial cells is a specific histological marker of pAMR. Currently primary categories of pAMR are 0, H+, I+, pathologic and severe pathologic AMR [29], including C4d complement deposition. Numerous studies have suggested that C4d and DSA may not be sufficiently sensitive markers of AMR [29, 33]. Although de novo HLA DSA associate with poor survival [34], and CAV [35-37], HLA DSA may not represent a reliable independent indicator of AMR for several reasons. Non-HLA autoantibodies may damage allograft endothelium and induce AMR in the absence of HLA DSA [38-40], and serum antibody levels fluctuate due to therapeutic removal and graft absorption. Several gene- and protein-based studies were recently conducted to find biomarkers for AMR and CAV with limited success [41-43]. These reports highlight the inconsistency of C4d and DSA as indicators for AMR and emphasize the urgency for additional diagnostic markers.

In this study, we assessed the correlation of pAMR with HLA antibody-mediated capillary EC activation, measured by phosphorylated S6K, S6RP, and ERK, in cardiac biopsy tissue. Our data show a strong independent association between pAMR/C4d and expression of p-S6K and p-S6RP in capillary EC ($p < 0.001$ for both p-S6K and p-S6RP). In addition, positive staining of capillary phosphorylation of both S6K and S6RP strongly correlates with the diagnosis of pAMR. This study confirmed previous finding of an association between pAMR and capillary phosphorylated S6RP [26]. The results herein further expand on this work by showing that phosphorylated S6K is also predominant in grafts from patients with AMR, and by demonstrating that C4d negative pAMR can be detected by p-

S6K staining. These results are also consistent with a recent study [44], wherein S6RP and p70S6K phosphorylation closely associate with AMR grade, DSA and microvascular inflammation. In our study's cohort, the incidence of DSA was significantly higher in C4d+/pAMR+ group than other groups. However, phosphorylation of S6K was significantly associated with pAMR irrespective of C4d staining. The trend toward an association of ERK with C4d+/pAMR- status requires further investigation with larger patient numbers.

Our results suggest that activation of cell proliferation pathways in graft capillary endothelium, such as phosphorylation of S6K, would be a valuable adjunct indicator for ambiguous AMR. We observed *in vitro* that HLA antibody binding to endothelium activate intracellular signaling cascades [21, 23, 25, 26, 45]. The current findings indicate that capillary endothelial activation occurs in response to endothelial interaction with HLA antibodies *in vivo*. We have shown that mTOR- and ERK-related signaling is important for endothelial proliferation *in vitro* and in murine models [21, 23, 25], which might contribute to the process of chronic rejection. It is notable that mTOR is required for endothelial survival signaling after HLA crosslinking [23, 46], suggesting these pathways may be cytoprotective as well [23, 24, 47, 48].

The molecular mechanisms underlying AMR are poorly understood. Classically, antibody induces capillary injury through Fc-mediated effects, activating the complement cascades [49] and recruiting leukocytes [50, 51]. Numerous *in vitro* studies by our group and others have also established that clustering of MHC class I molecules on vascular EC by anti-HLA antibodies directly triggers cellular activation leading to proliferation, stress fiber formation, and migration through activation of kinases such as FAK, paxillin, PI3K, Akt, mTOR, S6K, and S6RP [21, 23, 45, 46]. S6K and S6RP have been implicated as important regulators of mammalian cell size, protein synthesis, mRNA processing, glucose homeostasis, cell proliferation and survival [52, 53]. These pathways are activated in endomyocardial biopsies of cardiac transplant recipients, where phosphorylated S6RP in the capillary endothelium strongly associated with DSA to class II and AMR [26]. S6K can stimulate protein synthesis through phosphorylation of S6RP, eukaryotic initiation factor 4B (eIF4B) and Eukaryotic elongation factor 2 kinase (eEF2K) [52].

Recently, we found that mTOR is a central regulator of HLA class I-induced signaling, and was required for proliferation [21, 23], cytoskeletal changes [22, 54], and phosphorylation of S6K and S6RP. These *in vitro* data suggest that therapies targeting the mTOR complex 1/S6K pathway could be utilized to treat chronic rejection caused by donor specific HLA antibodies. Indeed, the mTOR inhibitor everolimus, which inhibits S6K phosphorylation *in vitro* [25, 55], has been recently investigated for the prevention of TCAD in heart transplantation [56, 57]. We speculate that mTOR inhibition will reduce the incidence of TCAD in the presence of DSA, due to inhibition of mTOR-dependent proliferative signaling.

In summary, we found that intragraft endothelial phosphorylation of S6K and S6RP independently correlated with the presence of pAMR. Our results point to phosphorylated S6K and S6RP as effective histological markers of pAMR even in the absence of C4d staining, and highlight the significance of HLA antibody-induced vascular signaling in the

process of graft injury. The phosphorylation of multiple cell proliferative proteins in heart allograft biopsies demonstrates that AMR is a dynamic and multifactorial pathological response. Finally, given that mTOR is a critical regulator of HLA I signaling in *in vitro* and *in vivo* models, further elucidation of the signaling cascades elicited during allograft rejection may identify new histological markers and therapeutic agents for the diagnosis and treatment of AMR.

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Figure 1.
Examples of immunohistochemical (IHC) staining of capillary endothelial cells: A) negative control; B) phosphorylated S6K stain; C) phosphorylated S6RP stain, and D) phosphorylated ERK stain (all immunoperoxidase IHC stains, orig mag x400).

Figure 2.

Capillary staining of phosphorylated S6K positively associated with the diagnosis of AMR. **A.** capillary phosphorylated S6K identifies both C4d+ and C4d- pAMR. The proportions of positive phosphorylation staining with score 2 or greater in C4d+ or C4d- pAMR- groups and C4d+ or C4d- pAMR+ groups were plotted. **B.** DSA was only strongly associated with C4d+pAMR patients. The proportions of DSA presence were plotted against the combinations of C4d staining and pAMR diagnosis.

Table 1

Patient Demographics.

Variables	C4d-/pAMR- (n = 29)	C4d+/pAMR- (n = 29)	C4d-/pAMR+ (n = 22)	C4d+/pAMR+ (n = 27)
<i>Gender</i>				
<i>Males</i>	21	19	12	18
<i>Females</i>	8	10	10	9
<i>Age, years (mean ± SD)</i>	43 ± 21	42 ± 25	39 ± 21	43 ± 21
<i>Time to biopsy, days (minimum – maximum)</i>	4 – 2761	3 – 1538	4 – 1374	6 – 2663
<i>Acute Cellular Rejection (ACR)</i>	8	10	8	8

Table 2

Associations among capillary phosphorylation S6K (2+), phosphorylation S6RP(2+), phosphorylation ERK(2+) and the classification of pAMR and C4d staining.

Phosphorylated staining	grade	C4d-/pAMR- (n = 29)	C4d+/pAMR- (n = 29)	C4d-/pAMR+ (n = 22)	C4d+/pAMR+ (n = 27) ^{**}	P value [*]
Capillary phosphorylated S6K	0	18	14	0	2	P<0.001
	1	11	10	9	12	
	2	0	3	8	9	
	3+	0	2	5	4	
Capillary phosphorylated S6RP	0	22	13	4	5	P<0.001
	1	7	11	9	13	
	2	0	5	3	4	
	3+	0	0	6	5	
Capillary phosphorylated ERK ^{**}	0	2	1	2	0	P<0.04
	1	8	1	5	9	
	2	13	14	7	12	
	3+	6	13	8	4	

* P value represents 'one-at-a-time' tests of significance for association between phosphorylation grades across the four classifications of pAMR and C4d staining.

** 2 patients lack phospho-ERK staining in C4d+/pAMR+ group due to insufficient tissue.

Table 3

Association between grades of staining of S6K, S6RP, ERK and pAMR

	Odds Ratio	p-value	95% CI
S6K, grade 0	Baseline	N/A	N/A
S6K, grade 1	18	0.001	3 – 100
S6K, grade 2	52	<0.001	6 – 425
S6K, grade 3+	49	0.001	5 – 521
S6RP, grade 0	Baseline	N/A	N/A
S6RP, grade 1	4	0.06	1 – 13
S6RP, grades 2, 3+	10	0.008	2 – 52
ERK, grade 0	Baseline	N/A	N/A
ERK, grade 1	5	0.2	0.4 – 53
ERK, grade 2	0.8	0.8	0.1 – 7
ERK, grade 3+	0.4	0.4	0.04 – 4

Table 4

Phosphorylation patterns of ACR biopsies within pAMR- and pAMR+ groups.

Phosphorylated staining	grade	pAMR- (n = 58)			Fisher exact test (p-value)	pAMR+ (n = 49)			Fisher exact test (p-value)
		NO ACR (N=40)	ACR1 R (n=15)	>ACR2 R (n=3)		NO ACR (N=33)*	ACR1 R (n=13)	>ACR2 R (n=3)	
Capillary phosphorylated S6K	Neg (0-1)	37	13	3	0.70	11	9	3	0.01
	Pos (2-4)	3	2	0		22	4	0	
Capillary phosphorylated S6RP	Neg (0-1)	36	15	2	0.16	20	9	2	0.88
	Pos (2-4)	4	0	1		13	4	1	
Capillary phosphorylated ERK	Neg (0-1)	11	1	0	0.24	13	3	0	0.26
	Pos (2-4)	29	14	3		18	10	3	

* 2 patients have no phospho-ERK staining due to insufficient tissue.