Journal of Clinical & Experimental Cardiology

McDaniel et al., J Clin Exp Cardiolog 2012, S:11
DOI: 10.4172/2155-9880.S11-002

Research Article Open Access

AIF-1 Gene Expression and Polymorphism in Association with Cardiac Allograft Rejection

D.O. McDaniel[1*], L. K. Piazza[2], Andrea Barker[1], H. Robertson[1], C. K. Moore[2], X. Zhou[3], P. Redmond[1], Y. Jackson[1] and G. Aru[1]

[1]*Department of Surgery, University of Mississippi Medical Center, Jackson, Mississippi, USA*
[2]*Department of Medicine, University of Mississippi Medical Center, Jackson, Mississippi, USA*
[3]*Department of Pathology, University of Mississippi Medical Center, Jackson, Mississippi, USA*

Abstract

Long- term clinical outcomes after cardiac transplantation still remains a challenge because of rejection episodes and the development of coronary vasculopathy. Rejection episode (RE) is a complex immunologic response, associated with the inflammatory signaling network. Allograft inflammatory factor-1 (AIF-1) has an important role in inflammatory process associated with transplantation. The goal was to demonstrate an association between the AIF-1 gene expression and genotype variation with RE. Peripheral blood and endomyocardial specimens were tested by semi-quantitative RT-PCR and immunohistochemistry (IHC) stains for identification of AIF-1 and IL-18 and were analyzed against clinical ISHLT grades for rejection. Sequence-specific primers for AIF-1 gene polymorphism were used to determine the C or the T allele variation in association with RE. The prevalence of CT heterozygous alleles were found significantly higher in patients who were presented with (0) RE during the first 6 months after transplantation as compared with CC alleles. The correlation of having CT alleles versus CC were inversely distributed with the increase in the number of RE. The isoform 2 expression was almost 2-fold higher than isoform 1 or isoform 3 in specimens with grade 3A RE versus specimens with grade 0-1 RE ($p<0.001$). The AIF-1 and the IL-18 were present in CMCs and in most of the MNCs in the specimens with grade 3A RE. The AIF-1 mRNA transcript expression was increased 5-fold in the biopsy specimens and it was1.7-fold higher than in peripheral blood monocytes in grade 3A RE. The IL-18 expression was increased 4.2-fold in biopsy specimens presented with 3A versus grade 0 (N) RE. Individuals with AIF-1 CC alleles are at a greater risk of developing the early rejection episodes. AIF-1 could serve as a suitable biomarker to monitor cardiac allograft RE through a less invasive procedure.

Keywords: African-American; Allograft inflammatory factor-1; Caucasian; Genotypes; Isoform; Rejection; Polymorphism; Transplantation

Introduction

Long- term clinical outcomes after cardiac transplant still remains a challenge because of infection, cellular rejection (CR) and the development of coronary vasculopathy (CV) [1,2]. Rejection episodes (RE), after cardiac transplantation is a complex immunologic response involving multiple signaling processes, associated with the inflammatory network. The most common rejection is acute CR, associated with T cell response and massive infiltration of the mononuclear cells (MNs), causing early period death after transplantation [3]. However, the early mechanism triggers the rejection may involve activation of the recipient immune responses initiated during the organ recovery and procurement, causing the release of inflammatory cytokines, sustaining the cellular infiltration, and the progression of rejection [4,5]. Endomyocardial biopsy (EMB) has been used for decades as the main monitoring method for cardiac allograft rejection. However, EMB is invasive, it can create complications [6], it is inconvenient and expensive and focal biopsy sampling often misses patchy foci of rejection. One possible alternative to EMB could be a biomarker that reliably is detectable in both peripheral blood and biopsy specimen and provides evidence of rejection as well as clinical outcomes. To date, no reliable biomarkers have been identified to accomplish these tasks.

Our previous study using a Human Common Cytokine Gene Array demonstrated a superior expression levels of Allograft Inflammatory Factor-1 (AIF-1) associated with CV and clinical grade 3A/3B rejections. Additionally, various inflammatory cytokines of which IL-18 had the highest intensity followed by IFN-γ,-α; FGFs; TGF-β and TNF-α,-β were present in association with rejections [7]. It is thought that AIF-1 increased expression induces activation and proliferation of vascular smooth muscle cells (VSMC) in the media of injured vessels, which subsequently leads to intimal thickening, hyperplasia and progression to vasculopathy and cardiac dysfunction. Indeed, in a mouse model Autieri et al. has shown that over expression of AIF-1 promotes proliferation of vascular smooth muscle cells and cell migration [8].

AIF-1 is a 17-kDa cytoplasmic, Ca++-binding protein, inducible by several cytokines primarily by IFN-γ, IL-1β, IL-18 as well as T cell conditioned media [9,10]. This suggests a fundamental role of AIF-1 in the processing of the inflammatory responses. AIF-1 was originally identified and cloned from rat cardiac allografts with chronic rejection [11]. Subsequently, it was identified in human and was shown to have high amino acid homology to rat AIF-1 [12,13]. Since then it has been shown that AIF-1 is involved in many pathological processes and is expressed primarily in dendritic cells and macrophages [12].

***Corresponding author:** D. Olga McDaniel, PhD, Department of Surgery, University of Mississippi Medical Center, 2500 North State Street, Jackson, Mississippi 39216-4505, USA, Tel: (601)- 984-5081; Fax: (601)-984-6614; E-mail: omcdaniel@umc.edu

Received June 20, 2012; **Accepted** July 13, 2012; **Published** July 13, 2012

Citation: McDaniel DO, Piazza LK, Barker A, Robertson H, Moore CK et al., (2012) AIF-1 Gene Expression and Polymorphism in Association with Cardiac Allograft Rejection. J Clin Exp Cardiolog S11:002. doi:10.4172/2155-9880.S11-002

Furthermore, it has been found in many other type of cells including activated T cells [10,14], blood vessel smooth muscle cells after a balloon injury [15], a subset of brain microglial cells [16], vascular endothelial cells [17], devascularized skeletal muscle tissues [18], Langerhan's cells [19], umbilical cord blood stem cells [20], kidney podocytes [21], and spermatides [22]. AIF-1 expression is observed in human and animal CNS pathological conditions such as traumatic brain injury, gliomas, brain infarction, autoimmune encephalomyelitis, and tissues from patients with rheumatoid arthritis and systemic sclerosis [23,24]. We and others have demonstrated that the persistent presence of AIF-1 in endomyocardial biopsies at posttransplantation was associated with grade 2 and 3 CR [25,26]. The positive staining of the AIF-1 in immunohistochemistry test was localized to infiltrating mononuclear cells in the interstitial and perivascular spaces of the allograft [26].

In humans, the AIF-1 gene maps to the major histocompatibility complex class III region on chromosome 6p21.3. This is a region which is densely clustered with genes associated with the inflammatory responses, including TNF-α/β, HSP and NF-κB [27]. The AIF-1 gene has six different exons that are differentially spliced to produce at least three different variants [28] (accession numbers: NM_032955, NM_004847, and NM_001623). The variant 1 encodes isoform 1 and has an identical C-terminal with the variant 3 (isoform 3). The variant 2 encodes isoform 2, and differs in the 5´UTR, thus has a different C-terminal, than isoform 1 and 3. The variant 3 encodes isoform 3 and is the longest isoform among the three isoforms. Several single nucleotide polymorphisms (SNPs) have been identified in the AIF-1 gene, but not all are located in all isoforms. A detailed location-description of the variants and the SNPs is given by Otieno et al. [29]. Two of these SNPs are in variant 2 (rs2269475, C→T and rs1315276, C→T) and one in variant 3 (rs2736182, G→A). The others (rs2844475, rs4711274, rs2736181, and rs2259571) are located either in the introns or the promoter region of the gene. Only rs2269475 in variant 2 was shown to be associated with systemic sclerosis and rheumatoid arthritis [29,30], and it was suggested that such polymorphism in the gene may represent the initiating event associated with the pathogenesis of the disease. A non-synonymous polymorphism within the promoter region of the human AIF-1 gene, defined by Genbank accession number AF097515, characterized as a C→T substitution at position –932, was reported to be more prevalent in cardiac transplant recipients as compared with a control population [31]. Since common to systemic sclerosis, rheumatoid arthritis, and cardiac rejection is an activated immune response, that triggers release of inflammatory mediators and cytokines, we undertook an analysis of the gene polymorphism and the expression levels of AIF-1 isoforms in patients that had undergone cardiac transplantation. The goal was to identify variations between the isoforms or alleles associated with allograft rejection. We also examined co-expression of IL-18 which plays a significant role in ischemia/reperfusion induced myocardial injury [32], as well as enhancing aortic atherogenesis through the release of IFN-γ [33] and, is associated with acute kidney allograft rejections [34].

Materials and Methods

Patients

Informed consent was obtained as part of the protocol approved by the University of Mississippi Medical Center Review Board for obtaining blood samples and biopsy specimen from each patient. Specimens from 35 African-American and 85 Caucasian patients who had undergone cardiac allograft transplantation were studied. Blood and biopsy samples were obtained at the same time. Patients were classified into testing groups based on endomyocardial biopsy and histopathological assessments for rejection scores: Grade 0 (0R), Grade 1A (R), Grade 2, Grade 3A (2R), and 3A/3B (3R) according to an established criterion by the International Society for Heart and Lung Transplantation (ISHLT) [3]. For genotype association study, patients were grouped into 6 categories on the basis of the frequency occurrence of rejection episodes (RE) within the first 6 months after transplantation. It was assumed that if there was any functional association between the alleles and inflammation, the recipients that presented with multiple RE might vary in the allele distribution as compared with those with 0 or minimal numbers of RE during the early period of transplantation. The categories included: (0) RE; (0-1) RE; (1-2) RE; (2) RE; (2-3) RE and (≥) RE. The controls (77 African-American and 43 Caucasian) were from the same geographic area, with no history of hypertension, diabetes, renal failure, vascular diseases, stroke, or cardiomyopathy.

AIF-1 genotype determination

The polymorphism was determined based on the definition by Genbank accession number AF097515 that describes a C→T single base-pair substitution at position–932. For detection of variant alleles (C or T) we used a modification of primers previously reported [31]. Such modification in the primers allowed detection of the presence or absence of an amplified DNA fragment in the subjects possessing the C or T genotype without a need for restriction enzyme (RE) digest (Figure 1). The forward primer for amplification of C or T was 5´-gta-gat-gga-gcc-ctg-ggc-ag-3´, and the reverse primer was set to present the substitution, C or T (underlined): 5'-atg-cct-gca-tgt-gtg-cac-gca-cgc-3' or 5'-atg-cct-gca-tgt-gtg-cac-gca-cgt-3'. DNA was amplified in a total volume of 12.5 µl reaction mixture by an initial cycle of 94ºC for 2 minutes, followed by 25 cycles of 94ºC for 30 sec, and 72ºC for 60 sec. The amplified DNA fragments were visualized by 2% agarose gel, and the genotypes were determined based on the presence or absence of the target DNA fragments. However, each allele was additionally confirmed by DNA amplification and PML-1 (g^tgcaa) RE digestion demonstrated in Figure 1. By this method, substitution of the C→T generated PML-1 site which was identified by two fragments of 227-bp and 187-bp. The primers for amplification of a generic fragment, followed by digestion with PML-1 RE were at flanking regions from-1133 to -719: forward sequence, 5'-gta-gat-gga-gcc-ctg-ggc-ag-3' and reverse sequence, 3'-aac-gta-acc-ctc-tca-tct-tat-ct- 5' producing a 414-bp fragment.

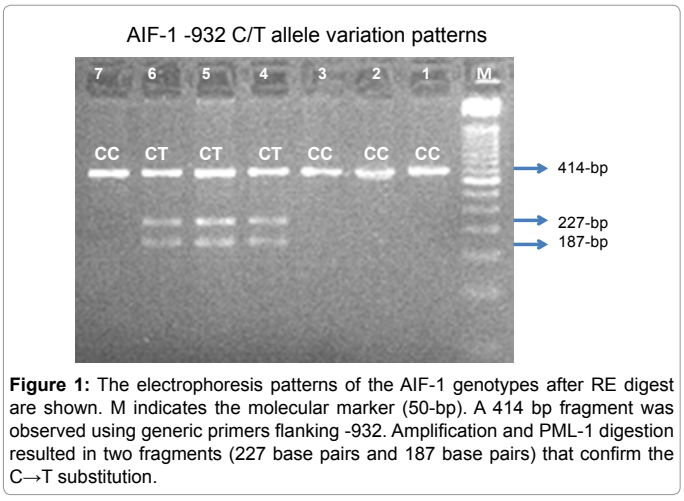

Figure 1: The electrophoresis patterns of the AIF-1 genotypes after RE digest are shown. M indicates the molecular marker (50-bp). A 414 bp fragment was observed using generic primers flanking -932. Amplification and PML-1 digestion resulted in two fragments (227 base pairs and 187 base pairs) that confirm the C→T substitution.

RNA isolation and reverse-transcription polymerase chain reaction (RT-PCR)

To determine AIF-1 and IL-18 mRNA transcript levels, RNA was isolated from peripheral blood mononuclear cells (PBMCs), monocytes and EMB specimens. Briefly, monocytes were isolated from patient's peripheral blood mononuclear cells (PBMCs) after a Ficoll-paque density gradient and positive selection for CD14-positive cells with Dynal beads. Cells were immediately homogenized in TRIzol and were stored at -80°C for subsequent RNA extraction. RNA was extracted from the cells using a kit from Promega (Promega Incorporation, Madison, WI), according to the guidelines. RNA concentration and purity were determined by RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA). Also, frozen biopsy specimens were homogenized in 200 µl cell lysis buffer for RNA extraction using a kit from Invitrogen (Invitrogen Corp. Carlsbad, CA, USA), according to the manufacturer's guidelines. Afterwards RNA was transcribed by ImProm-II Reverse Transcription method (Promega Incorporation, Madison, WI), followed by a routine RT-PCR protocol using primers for AIF-1 isoforms. The isoforms are generated due to the gene splicing. The isoform 1 has an identical C-terminal with the isoform 3, and the isoform 2, is different in both terminals with isoform 1 and isoform 3. However, they all share sequence homology. The orientation of the three isoforms is given in Figure 2 (a). The 5′ forward primers for Isoform 1 and 2 were identical, and the 3′ reverse primers for isoform 1 and 3 were identical. The forward primer for isoforms 1 and 2 was 5′- ATG-GAG-TTT-GAC-CTT-AAT-GG-3′, and for isoform 3, it was 5′- ATG-AGC-CAA-ACC-AGG-TAC-AG-3′. The reverse and complementary primer for isoforms 1 and 3 was 3′-GCA-ACT-CAG-AGA-TAG-CTT-TG-5′, and for isoform 2, it was 3′-TCA-CAT-TTT-TAG-GAT-GGC-AGA-TC-5′. The forward primer for IL-18 was 5′- ATG-GCT-GCT-GAA-CCA-GTA-g-3′, and for reverse and complementary, it was 3′-CTA-GTC-TTC-GTT-TTG-AAC-A-5′. The amplified PCR products for expression levels mRNA transcripts were visualized by 2.0% agarose gel. The intensity of the amplified banding pattern for each gene was quantified using a GDS-8000 (UVP) Image Analysis System and the level was normalized against the β-Actin gene. The normalized values (Unit) were presented as mean ± SEM.

Immunohistochemistry (IHC)

Surgical slides corresponding with the time of blood collection for RNA analysis were obtained from the Department of Pathology at the University of Mississippi Medical Center. The sections embedded in paraffin were deparaffinized in Xylene, and rehydrated through a graded series of ethanol followed by treatment with 3% hydrogen peroxide in phosphate buffered saline (PBS) to block the endogenous peroxidase activity. After antigen retrieval with Citra plus and blocking with normal serum provided in ABC kit, sections were incubated with primary antibodies against AIF-1 [26] or IL-18 [35] for one hour at room temperature. Then the sections were treated with biotinylated secondary antibody, followed by avidin-biotin peroxidase using Vectastain Ellite ABC kit (Vector Laboratories). Color development was visualized with DAB chromogenic substrate (Vector Laboratories), which was a reddish-brown stain followed by counterstaining with hematoxylin.

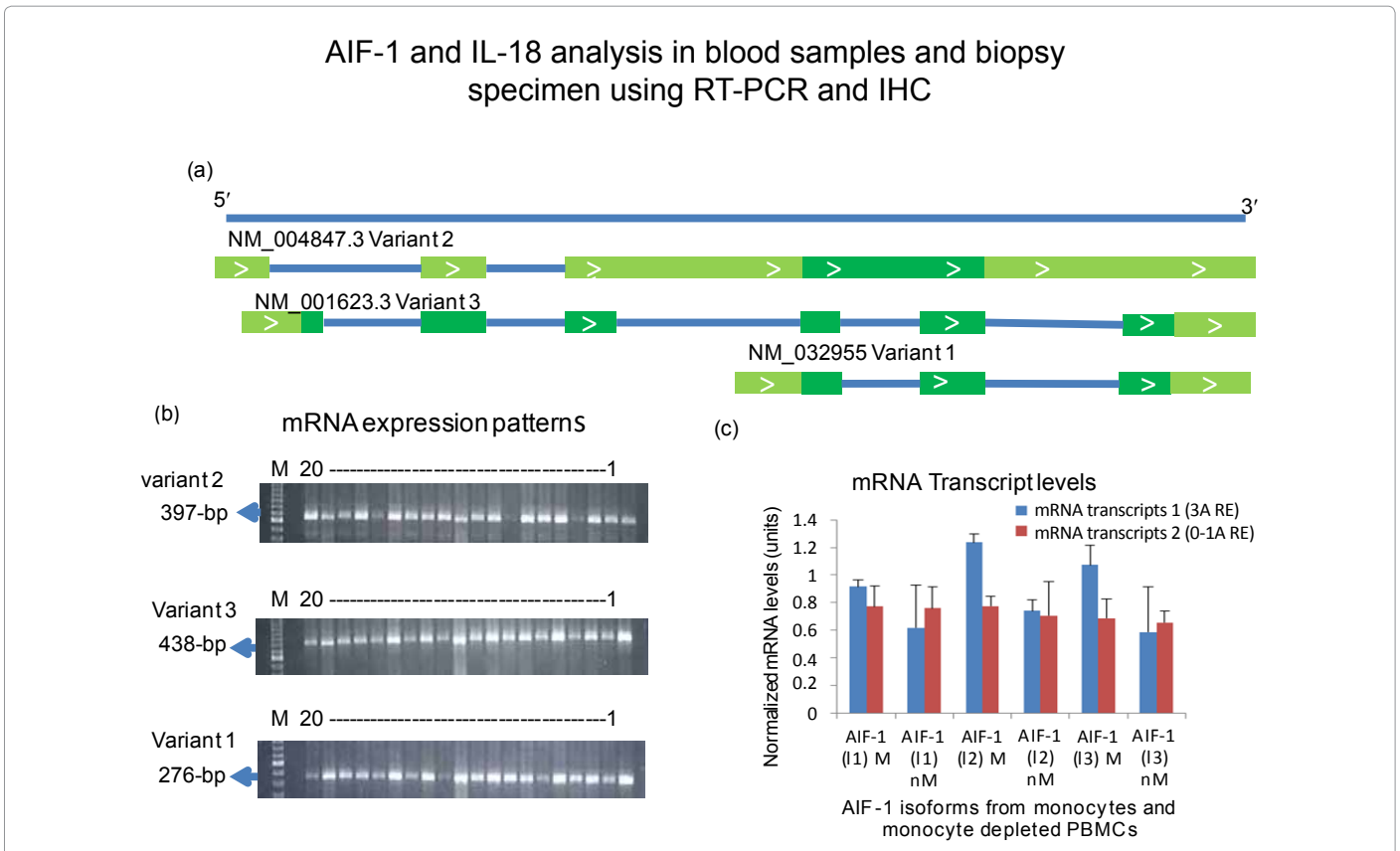

Figure 2: A diagram of AIF-1 variants (isoforms) and mRNA transcripts is illustrated. (a): the three isoforms have a partial sequence homology. The isoform 1 has an identical C-terminal with the isoform 3, and the isoform 2, is different in both terminals with isoform 1 and isoform 3. (b): mRNA expression pattern for the three isoforms is illustrated. 1 to 20 indicate patients samples, of which odd numbers represented mRNA tested from monocytes and even numbers mRNA from monocyte depleted PBMCs. (c): mRNA transcript levels calculated after they were normalized to β-Actin expression levels.

Data analyses

Allele frequencies of the genotypes were compared using contingency 2 x 2 table of Fisher's exact test. A P value less than 0.05 was considered statistically significant. The RNA transcript results were expressed as mean ± SEM. Group differences among mRNA transcript levels between the rejection grades were determined by one-way analysis of variance. Both interstitial infiltrating MNCs and CMCs were evaluated for IHC staining. Sections were analyzed for the presence and absence of AIF-1 and IL-18, in relation with pathological grades. The level of significance was set at $p<0.05$ for comparison between the groups.

Results

A total of 120 patients who had undergone cardiac allograft transplantation were investigated for AIF-1 allelic variation and AIF-1 gene expression in association with allograft rejection. There were 35 African-American and 85 Caucasian cardiac allograft recipients. The majority of the patients were Caucasian and male (73% and 76%, respectively). Based on pre-transplantation clinical diagnosis, the overall etiology of heart failure in this group of patients, for Caucasian recipients was ischemic cardiomyopathy, whereas 55% of African-American patients had been diagnosed with viral-mediated or idiopathic heart failure.

AIF-1 gene polymorphism associations

In a global analysis, shown in Table 1, the –932, CC homozygous genotype was approximately equally distributed in transplant recipients and the corresponding controls. The –932C alleles were the predominant genotype in both African-American and Caucasian populations. The distribution of the heterozygous C/T genotype was equivalent in both allograft recipients and controls, but the frequency was increased by 2-fold in Caucasians as compared to African-Americans. However, the difference was not statistically significant (African-American recipients: 11.4% vs. Caucasian recipients: 23.5%). The T/T genotype was absent in the African-American population and was present in a low frequency in the Caucasian population in this study. We further tested the allele distribution according to the 6 categories of patients as described in the methods. As shown in Table 2, the CT alleles were found significantly at a higher frequency in recipients who were presented with 0 number of rejection episodes during the first 6 months after allograft transplantation, as compared with those patients who carried homozygous CC alleles (CT:78.6% vs. CC:21.4%, $p<0.007$). The correlation of having CT alleles vs. CC were inversely distributed with the increase in the number of RE ($p<0.005$, Odds Ratio (OR) =18.3, 95% CI, (1.92-247)). This association may suggest that occurrence of RE frequently during the early months after transplantation might predispose a recipient to the inflammatory responses that are involved in the development of RE.

	Patients N=120		Controls N=120	
Genotypes	African American N=35, +(%)	Caucasian N=85, +(%)	African American N=77, +(%)	Caucasian N=43, +(%)
CC	31(88.6)	64(75.3)	63(81.8)	31(72.1)
CT	4(11.4)	20(23.5)[a]	14(18.2)	10(23.3)
TT[b]	0	1(1.2)	0	2(4.6)

a: CT genotype was 2.1-fold increase in Caucasian patients vs. African American but was not statistically significant. b: TT genotype was absent in African American patients and controls in this study, it was present with a low frequency among Caucasian patients and healthy controls.

Table 1: Genotype frequency distribution of AIF-1 polymorphisms in transplant patients and healthy controls

Alleles	CC	CT	P value
Rejection episodes	+ (%)	+ (%)	
0 (N=14)*	3 (21.4)	11 (78.6)	<0.007
0-1(N=27)	12 (44.4)	15(55.6)	NS
1-2(N=31)	23 (74.2)	8 (25.8)	<0.0001[a]
2 (N=18)	14 (77.8)	4 (22.2)	<0.002[b]
2-3(N=29)	23 (79.3)	6 (20.7)	<0.0001[c]
≥4 (N=12)*	10 (83.3)	2 (16.7)	<0.003[d]

a: Odds Ratio (OR)=8.26 (95% CI; 2.3-30.9); b: OR= 12.25 (95% CI; 2.05-85.6); c:OR=14.69 (95% CI; 3.5-66.35); d: OR=25.0 (95% CI; 2.16-484.8).

*: Individuals carrying CT alleles were compared in those with 0 rejection episode vs. ≥4 grade 3R cellular rejection episodes within the first 6 months after transplantation ($p<0.005$; OR=18.3; 95% CI; 1.92-247.02).

Table 2: AIF-1 allele frequency association with rejection episode patterns with the first 6 months after cardiac transplantation.

AIF-1 mRNA transcript evaluation

The resulting mRNA transcripts for the 3 isoform of AIF-1 were normalized to β-Actin expression levels and were compared in monocytes and monocyte depleted PBMCs. The normalized values (unit) were presented as mean ± SEM. Initially, to determine variations between the expression levels of the 3 isoforms in association with RE, RNA from 10 different recipients who were presented with grade 3A RE were tested. Furthermore, an additional 10 samples from the same recipients but with the sampling-time corresponding to the time that their myocardial biopsy results were grade 0-1A were tested. Figure 2 (b), shows fragment patterns of the three AIF-1 isoforms confirming the length of each variant. The resulting data in Figure 2 (c) indicated that the levels of AIF-1 mRNA expression was higher in monocytes as compared with monocyte depleted PBMCs. In addition only AIF-1 isoform 2 expression was significantly increased in association with grade 3A RE as compared with grade 0-1A. Isoform 3 was also increased in association with grade 3A RE but the difference was not statistically significant.

Subsequently, mRNA transcripts for AIF-1 isoform 2 expression were tested in monocytes and monocyte depleted PBMCs from a larger number of samples. A total of 45 patients were studied. This includes 15 patients in a group with normal biopsy (IHSLT grade 0 RE); 15 patients in a group with ISHLT grade 0-1A RE; and 15 patients in a group with ISHLT grade 3A (2R) RE. The results in Figure 3 (a) indicated that the AIF-1 isoform 2 was increased 1.8- fold and 1.5-fold in the group with 3A RE as compared with the group with grade 0 and grade 0-1A respectively ($p<0.001$). It was noted that the isoform 2 expression was not changed in monocyte depleted cells in association with RE grades. Furthermore, AIF-1 expression (Figure 3 b) was increased 5-fold in the biopsy specimen in grade 3A RE vs. specimens from grade 0 (N) and was increased 2.9-fold in specimens from grade 0-1A RE ($p<0.001$).

Since AIF-1 is inducible with IL-18 and the IL-18 is involved in cardiac ischemia/reperfusion injury, we then tested the levels of IL-18 mRNA expression in biopsy specimens. As shown in Figure 3 (b), the IL-18 expression was increased 4.2-fold in biopsy specimens from the group with grade 3A RE verses grade 0 (N), and 1.9-fold increased in the group with grade 0-1A RE ($p<0.05$).

Distribution of AIF-1 and IL-18 in biopsy specimens

We examined endomyocardial biopsy specimens from cardiac allograft recipients using the IHC protocol. To confirm specificity

of AIF-1 IHC stain, we tested the specimens with 3A RE with AIF-1 negative stain (Figure 3c, 1). There was no IHC signal in either MNs, or in CMCs. AIF-1 stain was present in cardiac myocytes (CMCs) and in most of infiltrating monocytes (MNs), in the specimens with grade 3A RE, shown in Figure 3 (c, 2). In CMCs, AIF-1 was found in the areas with both infiltrating MNs and without infiltrating MNs shown in Figure 3 (c, 2). In biopsy specimens presented with grade 3A RE, the IL-18 immunoreactivity was detected in monocytes but not in lymphocytes. Positive signals were present in all of infiltrating MNs and in CMCs. The absence of IL-18 was confirmed using a healthy heart tissue as a negative control. The IL-18 data is given in Figure 3 (c, 3 and c, 4).

Discussion

Evidence supports that molecular markers such as cytokines and inflammatory mediators play a crucial role in the development of acute as well as chronic rejection episodes after cardiac allograft transplantation [36]. Such posttransplantation clinical conditions are major contributing factors in long-term survival of cardiac allograft. Early blockade of such molecular markers may provide a beneficial effect on graft function and allograft survival. In this study, we investigated AIF-1 genotypes and expression levels of the isoforms in association with pathological RE grades in both peripheral blood and the biopsy specimens after cardiac allograft transplantation.

AIF-1, an evolutionarily conserved structural protein that has been implicated in regulation of inflammation, recently, re-discovered to be associated with cardiac allograft rejection after transplantation. The AIF-1 is expressed during ischemia/ reperfusion injury and that may play important role in the initial activation of the innate immune response, causing the release of inflammatory cytokines by the activated infiltrating cells residing in or entering the graft, leading to development as well as the maintenance of inflammatory responses after transplantation.

The current protocol for monitoring the survival of a cardiac allograft post-transplantation includes the testing of the biopsy samples at defined time intervals to evaluate indicators of rejection. Because these, procedures are invasive, they pose significant risks to the immunosuppressed transplant recipient. We have chosen testing of peripheral blood monocytes of allograft recipients because the proinflammatory cytokines such as IL-18 as well as AIF-1 are highly expressed on monocytes and macrophages on injury activated cells [26,35]. In addition, infiltrating mononuclear cells (MNCs) are present in myocardial tissues and coronary arteries due to the allograft rejection. Current study supports the presence of AIF-1 in allograft cardiomyocytes (CMCs) in the presence as well as absence of infiltrating MNCs [26], suggesting that the allograft CMCs are able to express the AIF-1. However, the role that AIF-1 plays in cardiac allograft rejection remains unclear.

Analysis of the mRNA transcript expression of the 3 isoforms in monocytes and monocyte depleted PBMCs from recipients, displayed that the isoform 2 expression was almost 2-fold higher than isoform 1 or isoform 3 in specimens with grade 3A rejection versus specimens with grade 0-1A rejections. This finding may have significant relevance in regards to the gene splicing nature of the isoform 2, and supports the previous report demonstrated the impact of AIF-1 isoform 2 on expression of the TGF-β cytokine and the pathogenic process in the endothelia and vascular alteration in systemic sclerosis [24]. Furthermore, studies using T cell cultures, confirmed the role of the isoform 2 and not isoform 3, that causing up-regulation of cytokines *in vitro* [10]. It was shown that the T cells expressing AIF-1 isoform 2, displayed significantly increased levels of IL-4 and IL-17 cytokines, whereas, the AIF-1 isoform 3 expressing T cells, demonstrated a slight

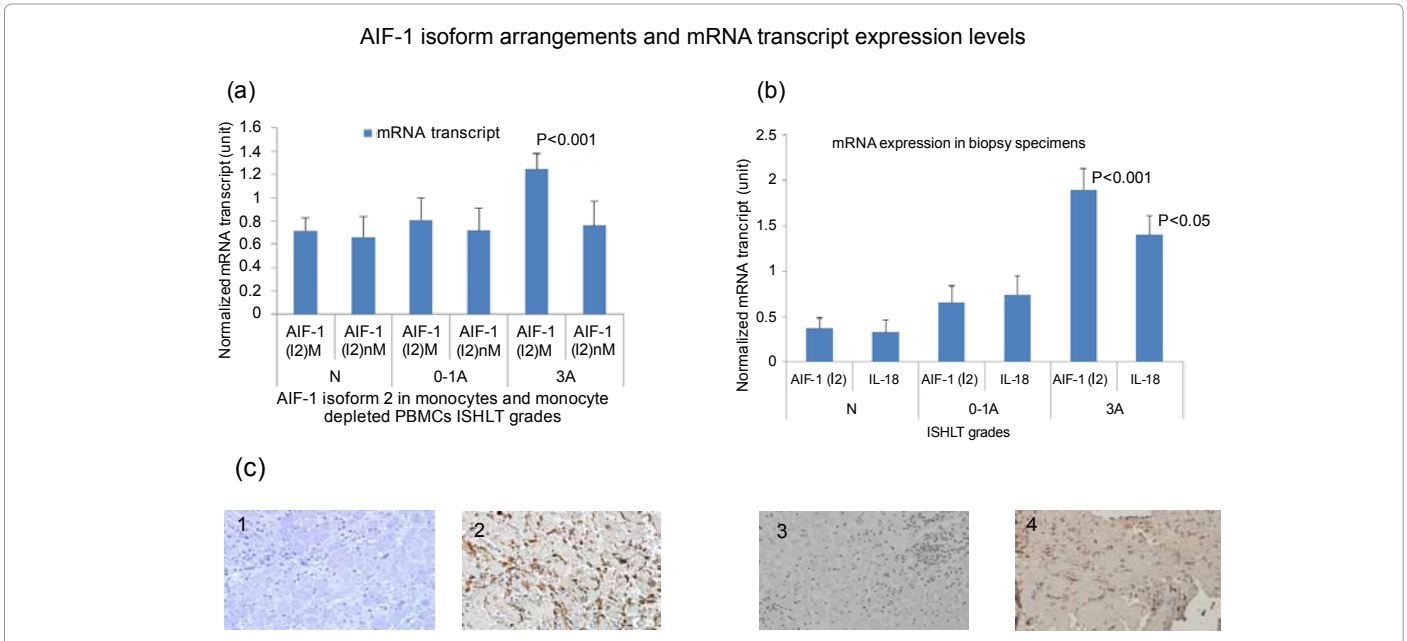

Figure 3: AIF-1 isoform 2 mRNA transcript and the IL-18 analysis in blood specimen and in the biopsy specimen are illustrated in sections (a), (b) and (c). (a): AIF-1 isoform 2 was increased in monocytes versus monocyte depleted PBMCs only in specimens that were presented with grade 3A RE ($p<0.001$). (b): AIF-1 and IL-18 mRNA transcript levels were highest in the specimens with grade 3A RE. (c): IHC stain with AIF-1 (1 and 2) and IL-18 (3 and 4) shows cross-sectioned CMCs and clusters of infiltrated MNs (40x). 1 is AIF-1 negative control, no IHC signal was seen either in CMCs or MNs. 2 is grade 3A (2R) RE, positive AIF-1 signals wre seen in some CMCs and in a majority of MNs infiltrated in the interstitial area. 3 is a negative control for IL-18 with no RE. 4 is a grade 3A RE, positive IL-18 were present in all infiltrating MNs but not in lymphocytes.

down regulation of these cytokines [10]. Studies reported increased expression of IL-4 and IL-17 in cardiac as well as renal allograft undergoing chronic rejections [37,38]. Furthermore, studies using condition media from AIF-1 isoform producing cells were shown to stimulate expression of the collagens, IL-6, TGF-β1, endothelin receptor and α-smooth muscle actin (α-SMA) in normal skin fibroblasts [10]. This indicates that the AIF-1 may have the potential for participating in the intracellular signaling, which leads to production of inflammatory molecules associated with RE after allograft transplantation.

Single Nucleotide Polymorphisms (SNPs) found in isoform 2 of the AIF-1 gene (rs2269475 and rs1315276), associated with systemic sclerosis and RA is another supporting evidence for the superior functional effects of the isoform 2 over the isoform 1 and isoform 3 in the process of inflammation [29,30]. Our earlier data failed to demonstrate an association between the rs2269475 SNP and the grade 3A rejection (data not shown). Therefore we analyzed the polymorphism reported by Turner et al. [31], that was found within the promoter region of the AIF-1 gene, defined by Genbank accession number AF097515, which presents a C→T single base pair substitution at position –932 [31]. This polymorphism has binding site for the E-box that has high affinity for the basic helix –loop-helix, family of transcription factors [39], which may have influence on expression of the AIF-1 protein. In the present study, there was no direct association between the AIF-1 alleles (CC, CT, and TT), generated due to the -932 substitution comparing patients with grade 0-3A RE and healthy controls. However, an association was found for the prevalence of C or T alleles when the recipients with grade 3A RE were placed into subgroups with variations in the frequency of the occurrence of RE during the first six months after transplantation (Table 2). We have shown that individuals carrying the CC homozygous alleles were at higher risk of developing grade 3 A RE. However, recipients who carried the CT heterozygous alleles were at minimal risk of experiencing RE more frequently than those patients with CC alleles during the first 6 months after cardiac allograft transplantation. It is not known whether frequent reoccurrence of RE during the early period after transplantation has any effects on the long term allograft survival, although, it could be suspected that it may alter immunosuppressive regimen or promotes an early vasculopathy and allograft dysfunction. The negative correlation between the T alleles and RE requires further investigation in a larger patient group. A correlation between the HLA-DR2 and the -932, T alleles has been reported. The HLA-DR2 is known to be a less responding genotype in a mixed lymphocyte cultures (MLC), suggesting that in a transplantation setting, the recipients carrying the HLA-DR2 genotypes are less responsive to the allograft, thus they experience less frequently rejection episodes during the early period after transplantation. The HLA genotypes were not the focus of this study however, it will be interesting to investigate whether the HLA-DR2 gene is in linkage disequilibrium with the genes regulating the AIF-1 production. The positive association between the C allele and the frequency of the RE may indicate a correlation between the genotype, AIF-1 production and the inflammatory processes leading to allograft rejection. The development of allograft rejection is associated with multiple genes that encode cytokines and growth factors such as IFN-γ, IL-10, IL-18, and TGF-β1, which may have influence in the regulation of a specific AIF-1 isoform. Co-expression of the IL-18 and the AIF-1, displayed in this study, suggest that a sequential activation of these molecules in association with inflammatory signaling network may uniquely regulate the innate immune responses, involved in organ transplantation. Ongoing studies are set to investigate whether the AIF-1, produced by cardiac myocytes are released into circulation in a timely sequence associated with the occurrence of pathologic changes. In addition, in vitro studies are in progress to investigate whether the AIF-1 plays as an endogenous molecule in the activation of Toll-like receptors in the pathogenesis of allograft rejection. An important highlight of this study was that the data presented a co-expression profile of AIF-1 and IL-18 that was correlated with biopsy-proven allograft rejection in both the peripheral blood and the biopsy specimens. This suggests that the AIF-1 could serve as a suitable biomarker to monitor cardiac allograft RE through a less invasive procedure. In addition, AIF-1 may have therapeutic potentials for strategies involved in the control of innate immune responses early on, after transplantation.

Acknowledgements

The authors thank the patients who participated in this study and the staff of the Cardiac Catheterization Laboratory at the University of Mississippi Medical Center (UMMC) for their assistance in obtaining blood samples and endomyocardial biopsy specimens. Also, the authors gratefully acknowledge the Genomic/Microarray Facility in the Department of Biochemistry at UMMC for the scanning and data analysis resources, Dr. Larry S. McDaniel, UMMC, for his critical review of the manuscript. This work was supported by Transplantation Immunogenetics Laboratory in the Department of Surgery at the University of Mississippi Medical Center as well as an Institutional Biomedical Research Funds awarded to Dr. Charles Moore.

References

1. Taylor DO, Stehlik J, Edwards LB, Aurora P, Christie JD, et al. (2009) Registry of the International Society for Heart and Lung Transplantation: twenty sixth official adult heart transplant report-2009. J Heart Lung Transplant 28: 1007-1022.
2. Kass M, Haddad H (2006) Cardiac allograft vasculopathy: pathology, prevention and treatment. Curr Opin Cardiol 21: 132-137.
3. Taylor DO, Edwards LB, Boucek MM, Trulock EP, Deng MC, et al. (2005) Registry of the International Society for Heart and Lung Transplantation: twenty-second official adult heart transplant report-2005. J Heart Lung Transplant 24: 945-955.
4. Millington TM, Madsen JC (2010) Innate immunity and cardiac allograft rejection. Kidney Int Suppl 119: S18-S21.
5. Wood KJ, Goto R (2012) Mechanisms of rejection: Current perspectives. Transplantation 93: 1-10.
6. Baraldi-Junkins C, Levin HR, Kasper EK, Rayburn BK, Herskowitz A, et al. (1993) Complications of endomyocardial biopsy in heart transplant patients. J Heart Lung Transplant 12: 63-67.
7. McDaniel DO, Yamout S, Aru G, May W, Thornton J, et al. (2004) Cytokine genes associated with CV following transplantation. International Proc Immunol Medimond 493-499.
8. Autieri MV, Carbone CM (2001) Overexpression of Allograft Inflammatory Factor-1 promotes proliferation of vascular smooth muscle cells by cell cycle deregulation. Atherioscler Thromb Vasc Biol 21: 1421-1426.
9. Liu G, Ma H, Jiang L, Zhou Y (2007) Allograft inflammatory factor -1 and its immunoregulation. Autoimmunity 40: 95-102.
10. Del Galdo F, Jimenez SA. (2007) T cells expressing allograft inflammatory factor 1 display increases chemotaxis and induce a profibrotic phenotype in normal fibroblasts in vitro. Arthritis Rheum 56: 3478-3488.
11. Utans U, Arceci RJ, Yamashita Y, Russell ME (1995) Cloning and characterization of allograft inflammatory factor-1: a novel macrophage factor identified in rat cardiac allografts with chronic rejection. J Clin Invest 95: 2954-2962.
12. Utans U, Quist WC, McManus BM, Wilson JE, Arceci RJ, et al. (1996) Allograft inflammatory factor-1. A cytokine responsive macrophage molecule expressed in transplanted human hearts. Transplantation 61: 1387-1392.
13. Autieri MV (1996) cDNA cloning of human allograft inflammatory factor-1: tissue distribution, cytokine induction, and mRNA expression in injured rat carotid arteries. Biochem Biophys Res Commun 228: 29-37.
14. Kelemen SE, Autieri MV (2005) Expression of allograft inflammatory factor-1 in T lymphocytes: a role in T-lymphocyte activation and proliferative arteriopathies. Am J Pathol 167: 619-626.

15. Autieri MV, Carbone C, Mu A (2000) Expression of allograft inflammatory factor-1 is a marker of activated human vascular smooth muscle cells and arterial injury. Arterioscler Thromb Vasc Biol 20: 1737-1744.

16. Deininger MH, Seid K, Engel S, Meyermann R, Schluesener HJ (2000) Allograft inflammatory factor-1 defines a distinct subset of infiltrating macrophages/ microglial cells in rat and human gliomas. Acta Neuropathol 100: 673-680.

17. Tian Y, Jain S, Kelemen SE, Autieri MV (2009) AIF-1 expression regulates endothelial cell activation, signal transduction, and vasculogenesis. Am J Physiol Cell Physiol 296: C256-C266.

18. Kuschel R, Deininger MH, Meyermann R, Bornemann A, Yablonka-Reuveni Z, et al. (2000) Allograft inflammatory factor-1 is expressed by macrophages in injured skeletal muscle and abrogates proliferation and differentiation of satellite cells. J Neuropathol Exp Neurol 59: 323-332.

19. Orsmark C, Skoog T, Jeskanen L, Kere J, Saarialho-Kere U (2007) Expression of allograft inflammatory factor-1 in inflammatory skin disorders. Acta Derm Venereol 87: 223-227.

20. Lund TC, Anderson LB, McCullar V, Higgins L, Yun GH, et al. (2007) iTRAQ is a useful method to screen for membrane-bound proteins differentially expressed in human natural killer cell types. J Proteome Res 6: 644-653.

21. Tsubata Y, Sakatsume M, Ogawa A, Alchi B, Kaneko Y, et al. (2006) Expression of allograft inflammatory factor-1 in kidney: A novel molecular component of podocyte. Kidney Int 70: 1948-1954.

22. Köhler C (2007) Allograft inflammatory factor-1/Ionized calcium-binding adapter molecule 1 is specifically expressed by most subpopulations of macrophages and spermatids in testis. Cell Tissue Res 330: 291-302.

23. Kimura M, Kawahito Y, Obayashi H, Ohta M, Hara H, et al. (2007) A critical role of allograft inflammatory factor-1 in the pathogenesis of rheumatoid arthritis. J Immunol 178: 3316-3322.

24. Del Galdo F, Maul GG, Jimenez SA, Artlett CM (2006) Expression of allograft inflammatory factor 1 in tissues from patients with systemic sclerosis and in vitro differential expression of its isoforms in response to transforming growth factor beta. Arthritis Rheum 54: 2616-2625.

25. Autieri MV, Kelemen S, Thomas BA, Feller ED, Goldman BI, et al. (2002) Allograft Inflammatory Factor-1 expression correlates with cardiac rejection and development of cardiac allograft vasculopathy. Circulation 106: 2218-2223.

26. Zhou X, He Z, Henegar J, Allen B, Bigler S (2011) Expression of allograft inflammatory factor-1 (AIF-1) in acute cellular rejection of cardiac allografts. Cardiovascular Pathol 20: e177-e184.

27. Iris FJ, Bougueleret L, Prieur S, Caterina D, Primas G, et al. (1993) Dense Alu clustering and a potential new member of the NF kappa B family within a 90 kilobase HLA class III segment. Nat Genet 3: 137-145.

28. Hara H, Ohta M, Ohta K, Nishimura M, Obayashi H, et al. (1999) Isolation of two novel alternative splicing variants of allograft inflammatory factor-1. Biol Chem 380: 1333-1336.

29. Otieno FG, Lopez AM, Jimenez SA, Gentiletti J, Artlett CM (2007) Allograft inflammatory factor-1 and tumor necrosis factor single nucleotide polymorphisms in systemic sclerosis. Tissue Antigens 69: 583-591.

30. Pawlik A, Kurzawski M, Szczepanik T, Dziedziejko V, Safranow K, et al. (2008) Association of allograft inflammatory factor-1 gene polymorphism with rheumatoid arthritis. Tissue Antigens 72: 171-175.

31. Turner DM, Pravica V, Sinnott PJ, Hutchinson IV (2001) Polymorphism in the promoter region of the gene encoding human allograft inflammatory factor-1. Eur J Immunogenet 28: 449-450.

32. Venkatachalam K, Prabhu SD, Reddy VS, Boylston WH, Valente AJ, et al. (2009) Neutralization of interleukin-18 ameliorates ischemia/reperfusion-induced myocardial injury. J Biol Chem 284: 7853-7865.

33. Whitman SC, Ravisankar P, Daugherty A (2002) Interleukin-18 enhances atherosclerosis in apolipoprotein-E (-/-) mice through release of interferon gamma. Circ Res 90: E34-E38.

34. Simon T, Opelz G, Wiesel M, Pelzi S, Ott RC, et al. (2004) Serial peripheral blood interleukin-18 and perforin gene expression measurements for prediction of acute kidney allograft rejection. Transplantation 77: 1589-1595.

35. Barker AK, McDaniel DO, Zhou X, He Z, Aru G, et al. (2010) Combined analysis of allograft inflammatory factor-1, interleukin-18, and Toll-like receptor expression and association with allograft rejection and coronary vasculopathy. Am Surg 76: 872-878.

36. Land WG (2005) Injury to allografts: innate immune pathways to acute and chronic rejection. Saudi J Kidney Dis Transpl 16: 520-539.

37. Mottram PL, Räisänen-Sokolowski A, Glysing-Jensen T, Stein-Oakley AN, Russell ME (1998) Cardiac allografts from IL-4 knockout recipients: assessment of transplant arteriosclerosis and peripheral tolerance. J Immunol 161: 602-609.

38. Loverre A, Tataranni T, Castellano G, Dirella C, Battaglia M, et al. (2011) IL-17 expression by tubular epithelial cells in renal transplant recipients with acute antibody mediated rejection. Am J Transplant 11: 1248-1259.

39. Hough PV, Mastrangelo IA, Wall JS, Hainfeld JF, Sawadogo M, et al. (1987) The gene-specific initiation factor USF (upstream stimulatory factor) bound at the adenovirus type 2 major late promoter: mass and three-dimensional structure. Proc Natl Acad Sci USA 84: 4826-4830.

This article was originally published in a special issue, **Cardiac Stem Cells** handled by Editor(s). Dr. Rosalinda Madonna, University of Texas Medical School, USA