Allograft Inflammatory Factor-1 Expression Correlates With Cardiac Rejection and Development of Cardiac Allograft Vasculopathy

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Background—Standard morphological features of endomyocardial biopsy specimens do not necessarily correlate with the efficacy of immunotherapy or development of cardiac allograft vasculopathy (CAV). We hypothesized that expression of allograft inflammatory factor-1 (AIF-1), a cytokine-inducible, calcium-binding protein associated with vascular smooth muscle cell proliferation, would be associated with allograft rejection and development of CAV.

Methods and Results—A total of 157 endomyocardial biopsy specimens from 26 patients with heart transplants were examined for expression of AIF-1 mRNA by semiquantitative reverse transcription–polymerase chain reaction. A significant relation was found between the International Society for Heart and Lung Transplantation rejection grade and expression of AIF-1 ($P<0.001$). The calculated odds ratio indicates that a biopsy has 2.5 times the chance of AIF-1 expression per grade of rejection. The relative concentrations of AIF-1 and GAPDH mRNA were calculated and the resulting ratios indicated that the amount of AIF-1 mRNA expression is relative to the rejection grade ($P<0.02$). In grade 1 biopsy specimens, AIF-1 was localized to infiltrating immune cells. In grade 3 biopsy specimens, AIF-1 was observed in immune cells and myocytes. AIF-1 is expressed in vascular and immune cells in coronary arteries with CAV, and persistent expression of AIF-1 in the allograft correlates with development of CAV ($P<0.002$).

Conclusions—Expression of AIF-1 in cardiac allografts correlates with rejection, and the amount of AIF-1 expressed correlates with the severity of rejection. AIF-1 is expressed in coronary arteries with CAV, and persistent expression of AIF-1 in the cardiac allograft is associated with development of CAV. (Circulation. 2002;106:2218-2223.)

Key Words: transplantation ■ restenosis ■ growth substances ■ biopsy

Despite advances in organ preservation and immunosuppressive therapy, the vascular narrowing indicative of graft vascular disease remains the major complication that limits long-term survival of heart transplantation.1 Treatment for this disease is difficult because it is a diffuse, concentric intimal hyperplasia extending throughout the coronary arterial tree, veins, and capillaries. Interventions that are successful in patients with conventional coronary artery disease are often not applicable to the majority of patients with cardiac allograft vasculopathy (CAV) because of its extensive nature. The pathogenesis of CAV is believed to involve a chronic immune response of the recipient to the donor vasculature in which activated recipient immune cells damage the endothelium, resulting in the production of cytokines, which elicit activation and proliferation of medial vascular smooth muscle cells (VSMC). The activation of VSMC is responsible for most of the obliterate arterial intimal thickening present in solid organ allografts as well as in CAV.1 The lack of an efficacious therapy to reduce or prevent progression of CAV emphasizes our current lack of understanding of the basic molecular mechanisms that promote this disease.

Data from several groups in diverse systems advocate an important role for allograft inflammatory factor-1 (AIF-1) in inflammatory processes. These studies range from expression in infiltrating macrophages in rat cardiac allografts; in lesions of experimental autoimmune encephalomyelitis; inflammatory lesions of the central nervous system; the pancreas of prediabetic BB rats; the allograft response of phylogenetically diverse species as marine sponges. AIF-1 transcript levels are significantly decreased in allografted animals that received immunosuppressive and immunomodulatory regimens, suggesting a tight association with the inflammatory process. Interestingly, a 44-amino acid segment of the AIF-1 protein contains an amidation signal and is flanked by a cluster of paired basic cleavage motif residues (-KR-KK-GKR), both of which are structural characteristics for peptide hormone precursor proteins.
We have previously reported the acute and transient expression of AIF-1 in medial and intimal VSMC in several models of arterial injury in rat and swine.\textsuperscript{11,12} Although constitutively expressed in leukocytes, AIF-1 is not expressed in unstimulated cultured human VSMC but is strongly induced in response to inflammatory cytokines and T-lymphocyte conditioned media.\textsuperscript{12} We have also shown that overexpression of AIF-1 in VSMC leads to a more rapid growth and deregulated expression of cell cycle proteins, suggesting that one function of AIF-1 might be in the regulation of VSMC proliferation.\textsuperscript{12,13}

Standard morphological features of endomyocardial biopsy specimens do not necessarily correlate with the development CAV.\textsuperscript{14} and a specific, reliable, objective, and rapidly quantitative surrogate is needed to detect the early development of CAV. We surmised that rejecting hearts would have variable degrees of activated VSMC and leukocyte infiltrate, whereas a nonrejecting heart should have neither. Because AIF-1 is expressed in the vasculature of rejecting hearts in cytokine-stimulated human VSMC and is constitutively expressed in leukocytes, we hypothesized that AIF-1 expression may reflect the rejection status of a heart transplant. The specific aims of this study are 4-fold: (1) to determine if AIF-1 mRNA expression is associated with rejection as measured by International Society for Heart and Lung Transplantation (ISHLT) grade, (2) to determine if the amount of AIF-1 expressed correlates with the severity of rejection, (3) to determine which cell or cells in the rejecting heart express AIF-1, and (4) to determine if AIF-1 expression is associated with development of CAV.

Methods

Patients and Myocardial Biopsy Samples

One hundred fifty-six endomyocardial biopsy samples from 24 patients with heart transplants were used in this study. These included both routine and biopsy specimens taken when rejection was suspected. Biopsy specimens were immediately frozen in liquid nitrogen, then stored at $-80^\circ$C for subsequent RNA extraction. Biopsy specimens were also taken for histology at the same time points and graded for cellular rejection by a pathologist blinded to other clinical or scientific data by using the criteria of the ISHLT.\textsuperscript{15} Additionally, all patients were treated with standard triple-therapy immunosuppression including cyclosporine, azathioprine, and prednisone. Episodes of moderate or severe rejection were treated with increased doses of prednisone or methylprednisone, ATGam, sol-umedrol, or OKT3, depending on the severity of the rejection and response to therapy. The Institutional Review Board approved all tissue procurement protocols for Human Studies at Temple University. Annual coronary angiography was used to assess CAV. CAV was compared with baseline levels and assessed for the presence of focal stenoses, distal tapering or pruning, and loss of tertiary vessels by cardiologists who were unaware of the studies on AIF-1.

RNA Isolation and Semiquantitative Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated from biopsies as described\textsuperscript{16} with Tri-Reagent (MRC Inc). Total RNA was reverse-transcribed with random hexamers and polymerase chain reaction (PCR)-amplified as described previously.\textsuperscript{12} Briefly, one fifth of the cDNA was PCR-amplified with the primers 5’ TAT CAT GTC CTT GAA ACG AAT GCC GGT AGA A 3’, and 5’ TTT GTC TTC TGT TTT AGC ATT CGG TCT CAG 3’, which define a 330-bp region of the human AIF cDNA, for 32 cycles. This is in the linear assay range with respect to cycle number, template concentration, and dilution of cDNA. Because of their small size, RNA from biopsy samples could not be quantified; thus, amplified cDNA was normalized to simultaneous amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH amplimers were purchased from Clonetech and define an amplicon of 450 bp. One fifth of the reaction was run on a 2.8% agarose gel, ethidium bromide–stained, and photographed. Some products were Southern transferred to hybridization membrane and hybridized with an end-labeled 45-mer oligonucleotide probe complementary for sequence internal to the PCR amplicons to verify AIF-1 amplification. The intensity of each band was quantified with image analysis software (NIH Image) and used to quantify AIF-1/GAPDH ratios.

Immunohistochemistry

Tissue sections were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm, deparaffinized in Xylene, and rehydrated through graded alcohols. Endogenous peroxidase activity was blocked with 1.5% hydrogen peroxide in methanol for 15 minutes. After blocking with normal serum, sections were incubated with primary antibodies for 1 hour at room temperature. CD31 (endothelial cell marker) and CD68 (macrophage marker) (Neo Markers, Inc) were used at a concentration of 2 μg/mL. SMC-α actin (Sigma) was used at 2-5 μg/mL, and AIF-1 antibody, which has been previously described, was used at 1.0 μg/mL.\textsuperscript{17} Sections were then incubated with biotinylated secondary antibody (1:200) followed by avidin-biotin-peroxidase complex in a Vectastain Elite kit (both from Vector Labs). The reaction product was visualized with DAB (Vector Labs) used as the chromogenic substrate, which produces a reddish-brown stain. The sections were counterstained with hematoxylin.

Data Analysis

Biopsy grade was obtained for each clinical specimen, and biopsy samples were analyzed for the presence or absence of AIF-1 expression. Generalized estimating equations for longitudinal data were used to analyze the relation between grade and presence or absence of AIF-1 (P<0.001). The odds ratio also indicates that a biopsy has a 2.5 times the probability of AIF-1 expression per grade of rejection. To correlate AIF-1/GAPDH ratio with grade, a normalized rank transformation of the data were analyzed with a mixed-model ANOVA for repeated measures followed by multiple comparisons to detect significant mean differences between grades. The Dunn-Bonferroni adjustment was used to maintain an experiment-wide type I error of ≤0.05, and differences between means were considered significant if the probability of chance occurrence was ≤0.05, using 2-tailed tests. χ² analysis was used to correlate AIF-1 expression with development of CAV.

Results

AIF-1 mRNA Expression Correlates With ISHLT Rejection Grade

Total RNA of quality and quantity sufficient to perform semiquantitative reverse transcription (RT)-PCR was obtained on 156 endomyocardial biopsies taken from 26 heart transplant recipients and examined for AIF-1 and GAPDH mRNA expression. These biopsy samples were obtained as routine surveillance or when clinically indicated. An example of AIF-1 mRNA detection by RT-PCR is presented in Figure 1A. Of the biopsy specimens tested, 56 were ISHLT grade 0, 61 were grade 1A or 1B, 18 were grade 2, and 21 were grade 3A or 3B, respectively. No grade 4 rejection was documented. A clear pattern of AIF-1 expression emerged as 13.5% of grade 0, 27.8% of grade 1, 48.5% of grade 2, and 69.8% of grade 3 were positive for AIF-1 (P<0.001) (Figure 1B). The relation between rejection score and AIF-1 mRNA
expression in 24 patients as determined by the calculated odds ratio indicates that a biopsy has a 2.5 times the probability of AIF-1 expression per increasing grade of rejection, indicating a significant correlation of AIF-1 mRNA with ISHLT rejection grade.

**AIF-1 mRNA Expression Is Proportional to ISHLT Rejection Grade**

The relative concentrations of AIF-1 and G3PDH mRNA from 76 biopsy specimens from 5 transplant recipients were calculated by scanning densitometry, and the resulting ratios of AIF-1/G3PDH indicate that the amount of AIF-1 mRNA expression is proportional to the rejection grade (Figure 2). The AIF-1 to GAPDH ratio during this period of time was 0.40, 0.63, 2.14, and 1.65 for grades 0 through 3, respectively (Figure 2B). The difference in ratio from a grade 0 to a grade 2 and a grade 1 from a grade 2 are statistically significant ($P<0.01$ and $P<0.02$, respectively). One biopsy specimen that scored a grade 0 and had an AIF-1/GAPDH ratio of 4.1 preceded a grade 3A biopsy specimen by 6 days.

Several of the patients whose biopsy specimens were graded 2 and more than half of the patients whose biopsy specimens were grade 3 were on increased immunosuppressive therapy (OKT3, ATGam, methylprednisone, high doses of prednisone) versus maintenance doses to counter acute rejection. Nine of the 11 biopsy specimens in which the AIF-1/GAPDH ratio was $<0.7$ were from patients receiving increased immunosuppressive therapy, suggesting that AIF-1 expression may mirror the efficacy of immunosuppression. This also probably accounts for the lack of statistical significance in ratio between a grade 0 and grade 3 biopsy specimen.

**Cellular Distribution of AIF-1 Expression**

To determine which cell or cell types were responsible for AIF-1 expression in endomyocardial biopsy specimens, we examined AIF-1 expression in endomyocardial biopsy specimens from transplant recipient hearts by immunohistochemistry. Similar to that observed by RT-PCR, very little to no AIF-1 immunoreactivity was detectable in the grade 0 biopsy specimens (Figure 3). In grade 1 to 2 biopsy specimens, AIF-1 immunoreactivity was localized to leukocytes. In grade 3 biopsy specimens, AIF-1 localized to leukocytes and cardiac myocytes.

**Correlation of AIF-1 Expression With CAV**

Longitudinal patterns of AIF-1 mRNA expression ranging in duration from immediately after transplantation to 932 days...
isolated from patients diagnosed with transplant arteriopathy. AIF-1 protein is strongly expressed in coronary arteries (both ischemic, and idiopathic dilated myopathy), and a normal donor. Western analysis showed that AIF-1 expression of coronary arteries from patients diagnosed with CAV, or rose again after this time, moderate to severe CAV was detected on 2-year angiography (Figure 4B). Patients with 2 or more episodes of elevated AIF-1 expression who had CAV at 1-year angiography had moderate/severe CAV at first-year angiography. Thus, persistent or episodic AIF-1 expression at more than 5 months after transplantation was determined in 108 biopsy specimens from 9 patients. One and two years after transplantation, coronary angiography was used to assess the development of CAV. Assessment of the longitudinal pattern of AIF-1/GAPDH ratios revealed two distinct patterns, examples of which are shown in Figure 4. Patients with an initial expression of AIF-1 that resolved within the first 5 months after transplantation did not have CAV, as determined by angiography at 1 and 2 years after transplantation (Figure 4A). However, if AIF-1 levels remained persistently elevated or rose again after this time, moderate to severe CAV was detected on 2-year angiography (Figure 4B). Patients with 2 or more episodes of elevated AIF-1 expression also had moderate/severe CAV at first-year angiography. Thus, persistent or episodic AIF-1 expression at more than 5 months after transplantation is associated with development of CAV (P<0.002).

**AIF-1 Is Expressed in Coronary Arteries With CAV**

To extend this analysis, we examined AIF-1 protein expression in coronary arteries from patients diagnosed with CAV, a patient with a stainless steel stent with in-stent restenosis, from patients with end-stage heart failure not due to coronary artery disease (both ischemic, and idiopathic dilated myopathy), and a normal donor. Western analysis showed that AIF-1 protein is strongly expressed in coronary arteries isolated from patients diagnosed with transplant arteriopathy as compared with arteries recovered from failing or normal hearts (Figure 5). AIF-1 is also highly expressed in the coronary artery with in-stent restenosis. We correlated expression of AIF-1 with proliferation by examination of Proliferating Cell Nuclear Antigen (PCNA) expression in extracts from these tissues. Comparable to AIF-1, significant PCNA expression was detected in arteries with arteriopathy but not from those isolated from failing or normal hearts, suggesting that AIF-1 expression is associated with proliferation of coronary artery cells.

It was important to determine the cellular distribution of AIF-1 in arteries with CAV. AIF-1 immunoreactivity is distributed in medial and neointimal α-actin–positive cells (Figure 6, A and E) as well as in CD31-positive cells (Figure 6C), indicating that VSMC and endothelial cells express AIF-1 in arteries with CAV. CD68-positive cells also express AIF-1 (Figure 6G), demonstrating that infiltrating macrophages express AIF-1 in these arteries as well.

**Discussion**

Graft surveillance by protocol biopsy is valuable in that it can directly reflect the immunologic status of the graft as well as the efficacy of antirejection therapy at a particular point in time. As such, the resulting ISHLT grade can be considered a risk factor for development of transplant arteriopathy. However, standard morphological features of endomyocardial biopsy specimens do not necessarily correlate with the development CAV, and a specific, reliable, objective, and rapidly quantitative marker is needed to detect the early development of CAV. We presumed that a nonrejecting heart would have neither a leukocyte infiltrate nor activated VSMC, as opposed to a rejecting heart, which would have varying degrees of both. Thus, AIF-1 would represent a sensitive, reproducible surrogate marker of transplant rejection.

Using generalized estimating equations for longitudinal data to take into consideration multiple biopsy specimens from individual patients, we found a significant relation between the ISHLT rejection grade and expression of AIF-1 (P<0.001). The calculated odds ratio indicates that a biopsy has 2.5 times the probability of AIF-1 expression per increasing grade of rejection. Similarly, in renal biopsy specimens, of several markers tested, including leukocyte surface antigens, cytokines, and activation markers, AIF-1 was the only marker able to distinguish subclinical from clinical rejection by immunohistochemical methods. Since subclinical rejection is a risk factor for renal graft dysfunction, this study suggested that AIF-1 might represent a marker of activated immune cells responsible for graft failure. Taken together,
these studies indicate that AIF-1 expression is a sensitive and specific marker of allograft rejection.

Quantification of AIF-1/GAPDH ratios in these biopsy specimens showed a strong correlation between the amount of AIF-1 detected and the severity of rejection. The difference in ratio from a grade 0 compared with a grade 2 and a grade 1 and grade 2 are statistically significant. The observation that more than half of the patients with grade 3 biopsy specimens were receiving rejection therapy is the most plausible reason for the grade 0 and grade 3 ratios not being statistically significant. Moreover, we observed a strong inverse relation between AIF-1 expression and immunosuppressant therapy. Similarly, in a mouse model of chronic allograft rejection, it was reported that AIF-1 mRNA expression could be modulated by therapy that suppressed the inflammatory response. Thus, examination of AIF-1 expression in a routine biopsy present earlier and more frequent opportunities to detect the effectiveness of immunosuppressive therapy. This is particularly valuable in that biopsy specimens examined in the first 3 months after surgery have been shown to demonstrate changes that can be tightly linked to subsequent development of CAV. In particular, the soluble products of immune cells that elicit rapid expression of AIF-1 in human VSMC are present in biopsy specimens within the first month of transplantation, before arterial lesions are detectable.

In a murine heterotopic model of chronic allograft rejection, a significant correlation was observed between development of intimal thickening and AIF-1 expression. Our analysis of sequential biopsy specimens in conjunction with angiographic analysis illustrated that patients with initially elevated levels of AIF-1, followed by a reduction to baseline levels, did not have CAV. However, in patients in whom AIF-1 levels increased after this initial rise and reduction, CAV did develop (P<0.002). This pattern is also similar to those observed for fibroblast growth factor (FGF) and thrombospondin-1 (TSP-1). In these studies, longitudinal analysis of FGF demonstrated that heart transplant recipients with late, elevated levels of FGF had an increased severity of CAV, whereas recipients who had low levels of FGF generally had no or mild CAV. A comparable observation was found with TSP-1, in that patients who had an initial elevation of TSP-1 followed by baseline levels had less severe CAV. In contrast, those individuals with a persistent elevation of TSP-1 had severe CAV. TSP-1 is analogous to AIF-1 in that it is induced in VSMC by inflammatory cytokines and TSP-1 induces VSMC proliferation. On this basis, taken together with our previous studies of AIF-1 expression, it is possible that increased AIF-1 expression will precede the morphological indications of CAV.

We have previously shown that AIF-1 expression is induced in several models of arterial injury, including rat aortic allografts and balloon-injured swine coronary arteries. In the current study, we extended this and showed that AIF-1 is induced by vascular as well as inflammatory cells in injured human coronary arteries and correlates with PCNA expression. The robust expression is localized to myofibroblast-like SMC α-actin–positive cells in the neointima. Since cultured VSMC only express AIF-1 when challenged with inflammatory stimuli including IFNγ, IL-1β, and TGFβ, cytokines elaborated by the large number of inflammatory cells in close proximity to these cells may be the source of the stimuli to the vascular cells.

Overexpression of AIF-1 in human VSMC leads to their increased proliferation, and stable expression in VSMC line leads to increased proliferation caused by shortening of the cell cycle by dysregulation of cell cycle proteins. Expression of AIF-1 in medial and neointimal cells and the relation between AIF-1 expression and development of CAV would confirm AIF-1 as at least a surrogate marker of graft rejection and may associate its expression with a more integral role in the pathogenesis of CAV.

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