Expression of Allograft Inflammatory Factor-1 Is a Marker of Activated Human Vascular Smooth Muscle Cells and Arterial Injury

Michael V. Autieri, Christopher Carbone, Anbin Mu

Abstract—The cytokine-induced activation and proliferation of medial vascular smooth muscle cells (VSMCs) leading to intimal hyperplasia is one of the most critical cellular events in the formation of transplant arteriopathy and balloon angioplasty-induced restenosis. Allograft inflammatory factor-1 (AIF-1) is a calcium-binding protein that we have previously shown to be expressed in balloon angioplasty–injured rat carotid arteries. We hypothesized that AIF-1 expression may be associated with the VSMC response to injury. In this study, we examined AIF-1 expression in immunologic and mechanical models of arterial injury. Reverse transcription–polymerase chain reaction and Western analysis demonstrated that AIF-1 is acutely and transiently expressed in aortic medial smooth muscle cells of rat cardiac allografts, with mRNA and protein peaking at 3 to 7 days after transplant and declining by 10 days after transplant. Immunohistochemical analysis identified abundant AIF-1 in the medial VSMCs of these vessels. Immunohistochemical analysis of balloon angioplasty–injured swine coronary arteries also demonstrates an acute AIF-1 expression detectable by 24 hours and continuing up to 14 days after the procedure. AIF-1 in these vessels also localizes to the medial VSMCs and cells of the developing neointima. AIF-1 protein is not expressed in quiescent cultured human VSMCs but is induced in cells challenged with various inflammatory cytokines, primarily by interferon-γ, interleukin-1β, and T-cell–conditioned media. Transfection and overexpression of AIF-1 in human VSMCs result in enhanced growth of these cells. Taken together, these data indicate that AIF-1 expression is associated with vascular trauma and suggest that this protein may play a role in VSMC activation subsequent to arterial injury. (Arterioscler Thromb Vasc Biol. 2000;20:1737-1744.)

Key Words: allograft inflammatory factor-1 ■ vascular smooth muscle cells ■ cytokines ■ transplant arteriopathy ■ balloon angioplasty

Vascular narrowing produced by graft vascular disease is a major complication that limits long-term survival of cardiac transplantation. A primary cause of cardiac allograft vasculopathy (CAV) subsequent to cardiac transplantation is the intimal proliferation of vascular smooth muscle cells (VSMCs) in large- and medium-sized arteries and veins. The initiation of CAV is believed to involve a chronic immune response of the recipient to the donor vasculature in which activated immune cells damage the endothelium, resulting in the production of cytokines. These cytokines in turn elicit the activation of medial VSMCs, which migrate into the intimal layer and proliferate. Similarly, the long-term efficacy of percutaneous transluminal coronary angioplasty as a treatment for advanced multivessel coronary artery disease is significantly limited by the high incidence of vascular restenosis observed in as many as 40% of the patients undergoing this procedure. Analogous to CAV, restenosis subsequent to balloon angioplasty injury is also a process mediated by activated VSMCs. It has been suggested that cytokine-induced activation and proliferation of medial VSMCs, leading to intimal hyperplasia, is the most critical cellular event in the formation of CAV and balloon angioplasty–induced restenosis. Accordingly, the activation of several selected genes may reflect the status of smooth muscle cell (SMC) activation and, thus, the development of arteriopathy indicative of the progression of neointimal hyperplasia. Identification and functional characterization of these gene products is a promising approach for the development of antirestenotic therapeutics.

Allograft inflammatory factor-1 (AIF-1) is a 143–amino acid calcium-binding protein. Initial investigations indicated that AIF-1 expression was limited to interferon-γ (IFN-γ)–stimulated macrophages and neutrophils, which infiltrated rat cardiac allografts. Our initial observations came from balloon angioplasty–damaged rat carotid arteries, in which we observed a rapid transient expression pattern in AIF-1 mRNA. The intimal proliferative response of balloon angioplasty–induced vascular injury in rats is similar to that

Received August 17, 1999; revision accepted January 31, 2000.
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observed in the rat cardiac transplant model with respect to endothelial cell loss, activation of medial VSMCs, and elaboration of neointimal hyperplasia. Considering the similarities between mechanical and immunologic injury, we hypothesized that AIF-1 would also be expressed in the VSMCs of allografted tissue and that its expression in CAV might be associated with the progression of vascular hypertrophy characteristic of balloon angioplasty–induced injury. It was our intention to focus our examination of AIF-1 expression to the SMC response to vascular injury, because VSMCs are the major cell type in the media and in the developing neointima in this tissue. In the present study, we examine AIF-1 expression in 2 in vivo models of arterial injury: immunologic (in rat aortic allografts) and mechanical (in porcine coronary artery overstretch). We also explore a more direct role for AIF-1 in human VSMC activation by examining its ability to influence VSMC growth. The goals of the present study are (1) to associate AIF-1 expression with arterial trauma in different models of arterial injury, (2) to compare this expression with progression of the restenotic lesion over time, (3) to localize AIF-1 expression to activated medial and intimal VSMCs, and (4) to characterize a role for AIF-1 in VSMC activation.

Methods

Heterotopic Rat Heart Transplantation

Lewis rats providing donor hearts were anesthetized with buprenorphine administered subcutaneously and 1% to 2% isoflurane gas inhalation. The hearts were removed and gently perfused with sodium heparin dissolved in saline and placed in a cold saline solution containing sodium heparin until transplantation. Recipient Wistar-Furth rats were similarly anesthetized, and the donor heart was transplanted into the recipient right abdominal cavity with the donor aorta connected to the recipient abdominal aorta and the donor pulmonary artery connected to the recipient inferior vena cava. Once completed, the wound was closed with sterile nonabsorbable sutures and swabbed with Betadine surgical scrub. At various times after transplant, the donor hearts were removed, and the aortas were isolated and freed of any adhesions and external material. Some samples were processed for RNA and protein isolation; others were fixed and processed for immunohistochemistry. All procedures were performed in accordance with guidelines approved by the Institutional Animal Care and Use Committee (IACUC).

Swine Coronary Artery Balloon Angioplasty

Domestic swine weighing 20 to 30 kg were sedated, and general anesthesia was induced. After an 8F introducer sheath with a hockey stick curve was placed in the right femoral artery of the anesthetized animals, they received a bolus of heparin (200 U/kg) and bretylium (2.5 mg/kg). A guiding catheter was advanced to the aortic root, intracoronary nitroglycerin (200 μg) was injected, and baseline angiograms of the left and right coronaries were obtained. Coronary injury was achieved by deliberate stretch of the vessel wall with an oversized angioplasty balloon inflated to a pressure of 8 to 10 atm for 30 seconds, with a 1-minute rest period, for a total of 3 times. After withdrawal of the catheter, the femoral sheath was removed, and the cutdown site was repaired. At various times after angioplasty, the animals were euthanized according to the standard protocol of massive pentobarbital overdose (100 mg/kg IV). The left main coronary artery was cannulated, and injured segments were located by examination, dissected in block, and processed for immunohistochemistry. All procedures were performed in accordance with IACUC-approved institutional guidelines.

Tissue Processing and Immunohistochemistry

Hearts excised from pigs were perfusion-fixed with 10% buffered formalin at physiological pressures (100 mm Hg), and injured segments were fixed in 10% formalin for 4 hours and then in 70% ethanol for 18 hours and paraffin-embedded. Rat aortas were dissected from the heart, cleared of adherent tissue, fixed, and processed as described for porcine tissue. Tissue sections were deparaffinized, rehydrated in PBS and ethanol, quenched with H₂O₂, and blocked with goat serum. For primary AIF-1 antibody, rabbits were immunized with a peptide corresponding to amino acids 17 to 33 (KAQSEQRELDEINNKFLHLH) present in the AIF-1 protein. Antibodies were affinity-purified with this peptide, and Western analysis of recombinant AIF-1 protein indicates that this antiserum recognizes a protein of the predicted 17 kDa. SMC α-actin antibody (Sigma Chemical Co) was used at a 1:2500 dilution to identify VSMCs. AIF antibody was applied at a 1:1000 dilution in PBS/1% BSA, washed, and incubated with biotinylated goat anti-rabbit secondary antibody (Transduction Laboratories) at 1:2000 dilution. This was washed and incubated with an avidin-biotin enzyme complex and chromogenic substrate as described by the manufacturer (Vectastain Elite ABC peroxidase), which develops a reddish-brown stain. Sections were counterstained with hematoxylin. Sections treated with secondary antibodies only or nonimmune IgG did not show any staining. Coronary vessels from at least 2 swine and 3 sections per vessel were evaluated.

Cells and Culture

Human coronary VSMCs were obtained as cryopreserved secondary culture from Clonetics Corp and subcultured in growth medium as described previously. Cells from passages 3 to 6 were used in the described studies. The growth medium was changed every other day until cells approached confluence. Preconfluent VSMCs were serum-starved (0.25% FCS) for 48 hours and then exposed to 10% FCS, 10 ng/mL basic fibroblast growth factor (bFGF), 100 U/mL IFN-γ, 20 ng/mL interleukin (IL)-1β, 20 ng/mL platelet-derived growth factor (PDGF)-AB, 2 ng/mL transforming growth factor (TGF)-β, or T-cell–conditioned medium for 48 hours, at which times samples were processed for protein isolation. Some samples remained untreated and were used as controls. PDGF, bFGF, IFN-γ, and TGF-β were purchased from Gibco-BRL; IL-1β was purchased from Boehringer-Mannheim; and T-cell–conditioned medium was purchased from Fisher Biotech.

Transfection and Proliferation Assay

The protein coding region of the AIF-1 cDNA was cloned by polymerase chain reaction (PCR) with the use of AIF-1 gene-specific primers. The 5′ PCR primer also contained a Kozak consensus sequence (GCCGCGCCATGG) to enhance translation. This was inserted into the expression vector pBK-CMV (Stratagene), and this purified DNA construct was termed pBK-CMV-AIF-1. Transfection of human VSMCs has been described. Briefly, human coronary artery SMCs grown in T-75 flasks were transfected with either no plasmid (mock control), pBK-CMV plasmid alone, or pBK-CMV-AIF-1 along with 3 μg/mL LipofectAMINE reagent (Life Technologies) and mixed with 1 μg/mL of either plasmid. Two days after transfection, the cells were trypsinized and split 1:2, with one half left to grow in the presence of growth medium +G418 (Genetin) for 15 days. The other half was saved for RNA isolation. After selection for 15 days, the cells were then trypsinized and counted by use of a standard hemocytometer.

Western Blotting

To prepare cell extracts, human VSMCs grown in T-75 flasks were cultured and treated as described above, washed with PBS, and treated with 0.3 mL of ice-cold lysis buffer (50 mmol/L HEPES-KOH, pH 7.5, 150 mmol/L NaCl, and 0.1% Triton X-100) containing protease inhibitors, as described. Lysates were incubated on ice 20 minutes, withdrawn through a 21-gauge needle 3 times, then centrifuged at 3000 rpm for 15 minutes at 4°C, and stored at −20°C. Proteins were extracted from rat aorta by Tri-Reagent (Molecular Research Center). Equal protein concentrations of cell extracts were electrophoresed through an 18% polyacrylamide gel under reducing conditions and transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat powdered milk in TBST buffer (0.1 mol/L Tris-HCl, pH 8.0, containing 1.5 mol/L NaCl and 0.5% Triton X-100) for 2 hours at room temperature. Blots were incubated with 1:1000 diluted antibody in TBST, washed 3 times, and incubated with horseradish peroxidase–conjugated secondary antibody. Chemiluminescent detection reagents (PerkinElmer) were used to visualize protein expression. Quantification used a densitometer (Molecular Dynamics). For all blots, similar intensity bands were observed for each antibody, indicating that comparable amounts of protein were loaded on each gel.
Expression of AIF-1 in VSMCs of Injured Arteries

Results

AIF-1 mRNA and Protein Are Expressed in Medial VSMCs in Allografted Rat Aorta

We initially examined the expression of AIF-1 mRNA in VSMCs subject to immunologic insult by using aortas isolated from rat cardiac allografts. Donor hearts from Lewis rats were heterotopically transplanted into the abdomen of Wistar-Furth recipient rats, and semiquantitative reverse transcription–PCR (RT-PCR) was performed on RNA isolated from aortas dissected from Lewis rat hearts allografted into Wistar-Furth hosts with use of human AIF-1 and G3PDH amplifiers defining amplicons of 330 and 450 bp, respectively. One fifth of the reaction was run on a 2.5% agarose gel, ethidium bromide–stained, and photographed. Lanes are as follows: lane 1, no reverse transcriptase (no RT) template (negative control); lane 2, naive Lewis heart; lane 3, 1 day after transplant; lane 4, 3 days after transplant; lane 5, 7 days after transplant; lane 6, 10 days after transplant; lane 7, 7-day syngeneic (Lewis into Lewis); lane 8, spleen (positive tissue control); lane 9, AIF-1 cDNA and G3PDH template (positive PCR control).

X-100). Membranes were incubated with a 1:2500 dilution of primary antibody and a 1:2000 dilution of goat anti-rabbit secondary antibody. Proliferating cell nuclear antigen and cyclooxygenase-2 antibody were from Santa Cruz, Inc. The membrane was washed with TBST, and reactive proteins were visualized by using the enhanced chemiluminescence method (Amersham) according to the manufacturer’s instructions.

RNA Isolation and Semiquantitative Reverse Transcription–PCR

For each time point studied, aorta from allografted rat hearts were isolated and cleared of adherent tissue. Total RNA was extracted by Tri-Reagent, which allowed simultaneous extraction of RNA and protein, and 4 µg of total RNA was reverse-transcribed by using random hexamers as described previously.14 One fifth of the cDNA was PCR-amplified by using the following primers: 5’TATTGTCCTTTGAAACGGATGGAGAA3’ and 5’TTTGGTCTTTGATTTAGCCTRCA3’, 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. The glyceraldehyde-3-phosphate dehydrogenase (G3PDH) amplimers were purchased from Clonetech and define an amplicon of 450 bp. One fifth of the reaction was run on a 2.5% agarose gel, ethidium bromide–stained, and photographed. PCR products were Southern-transferred to the hybridization membrane and hybridized with an end-labeled 45-mer oligonucleotide probe complementary for a sequence internal to the PCR amplicons. For verification of transfection efficiency, total RNA was extracted from transfected cells, and 4 µg was reverse-transcribed. One fifth of the cDNA was PCR-amplified by using neomycin-specific amplimers, which define a 290-bp region of the neomycin cDNA, for 32 cycles.

Figure 1. Expression of AIF-1 cDNA in aortas from allografted rat heart. Semiquantitative RT-PCR was performed on 4 µg total RNA isolated from aortas dissected from Lewis rat hearts allografted into Wistar-Furth hosts with use of human AIF-1 and G3PDH amplimers defining amplicons of 330 and 450 bp, respectively. One fifth of the reaction was run on a 2.5% agarose gel, ethidium bromide–stained, and photographed. Lanes are as follows: lane 1, no reverse transcriptase (no RT) template (negative control); lane 2, naive Lewis heart; lane 3, 1 day after transplant; lane 4, 3 days after transplant; lane 5, 7 days after transplant; lane 6, 10 days after transplant; lane 7, 7-day syngeneic (Lewis into Lewis); lane 8, spleen (positive tissue control); lane 9, AIF-1 cDNA and G3PDH template (positive PCR control).

Figure 2. Expression of AIF-1 protein in aortas from allografted rat hearts. Western analysis of protein from aortas dissected from Lewis rat hearts allografted into Wistar-Furth hosts by use of antibody directed to AIF-1 peptide sequence is shown. Lanes are as follows: lane 1, naive Lewis heart; lane 2, 1 day after transplant; lane 3, 3 days after transplant; lane 4, 7 days after transplant; lane 5, 10 days after transplant; lane 6, 7-day syngeneic (Lewis into Lewis); and lane 7, spleen (positive tissue control). Analysis of AIF-1 protein expression was detected by enhanced chemiluminescence.

Expression pattern of AIF-1 mRNA expression, with mRNA levels increasing within 24 hours of transplantation, peaking at 3 to 7 days, and declining by 10 days after surgery. Seven-day syngeneic (Lewis to Lewis) control aortas also demonstrate a low, but detectable, increase in AIF-1 mRNA expression. AIF-1 is constitutively expressed in spleen tissue and was used as a positive control. The identity of this amplicon as human AIF-1 was confirmed by Southern analysis of the same gel hybridized with a probe specific for a sequence internal to the AIF-1 amplifiers (Figure 1). These results indicate that AIF-1 mRNA is acutely and transiently induced in aortas from allografted rat hearts.

It was important to determine whether AIF-1 protein was expressed in a fashion similar to that for AIF-1 mRNA. Protein was extracted from the same aorta as used for RNA and subjected to Western analysis with AIF-1–specific antibody. Figure 2 indicates that AIF-1 protein expression is also inducible by cardiac allograft transplantation and follows a pattern similar to that for mRNA, with protein levels peaking at 3 to 7 days and declining by 10 days after surgery. No AIF-1 was observed 1 day after transplant, which may be due to the time necessary for mRNA to be translated into detectable amounts of protein. Again, a small increase in AIF-1 protein was detectable in 7-day Lewis to Lewis control rats. These results indicate that AIF-1 protein is also acutely and transiently expressed by immunologic insult and reflects that of mRNA expression.

We used immunohistochemistry to localize progression of AIF-1 protein accumulation in allografted aortas. Serial sections from Lewis to Wistar-Furth cardiac allografts were immunohistochemically examined with AIF-1 antisera, and Figure 3 shows rat aortas before and at various times after transplant. Similar to protein levels determined by Western analysis, little to no AIF-1 protein was present in the uninjured aorta or in the aorta 24 hours after transplantation (Figure 3A and 3B). By 3 days after surgery, however, a marked induction of AIF-1 protein was observed in the medial VSMCs, continuing through day 7 (Figures 3C and 3D). By day 10, AIF-1 protein levels appeared to decline, and a modest amount of AIF-1 protein was detectable in a 7-day Lewis to Lewis control aorta (Figures 3E and 3F, respectively).

It is important to note that positive AIF-1 staining localizes to SMCs, which are identified by their morphological features and positive staining for SM α-actin. (Figure 3H). These findings are also consistent with the
cellular pathology of this model, in which monocytes and macrophages are frequently found only in the allograft adventitia in early lesions and are not detected in the media and intima until 20 days to 3 months onward. Overall, these results closely mirror those observed for AIF-1 mRNA and protein expression as detected by RT-PCR and Western analysis and indicate that AIF-1 is expressed acutely and transiently in aortic VSMCs by allograft transplantation.

AIF-1 Protein Is Induced in Swine Coronary Arteries by Balloon Angioplasty

The swine response to vascular injury presents many similarities to that of humans, particularly in the generation of neointimal formation. We performed immunohistochemistry on swine coronary arteries subject to oversized-balloon angioplasty and examined them for AIF-1 expression at several time points after surgery. Figure 4A demonstrates that AIF-1

![Figure 3](image_url)

**Figure 3.** Immunohistochemical analysis of expression of AIF-1 protein in aortic VSMCs from Lewis rat hearts allografted into Wistar-Furth hosts. Cross sections of rat aorta (A, naive; B, 1 day after allograft; C, 3 days after allograft; D, 7 days after allograft; E, 10 days after allograft; and F, 7-day syngeneic [Lewis to Lewis]) were incubated with AIF-1 antisera. Panel G is secondary antibody only, and panel H is SMC α-actin. Red-brown staining indicates AIF-1 expression, and sections were counterstained with hematoxylin. Original magnification ×100.

![Figure 4](image_url)

**Figure 4.** Immunohistochemical analysis of expression of AIF-1 protein in VSMCs from balloon angioplasty–injured swine coronary arteries. Cross sections of balloon angioplasty–injured swine coronary arteries stained with AIF-1 antibody are shown: A, naive; B, 1 day after injury; C, 3 days after injury; and D, 14 days after injury. Panel E is secondary antibody only. Panels F to J are the same sections at higher magnification. Panels K to O are the same sections stained with SMC α-actin to localize medial VSMCs (m). n indicates neointima. Red-brown staining indicates positive staining, and sections were counterstained with hematoxylin. Original magnification ×40 (A to E) and ×100 (F to O).
protein was not detectable in uninjured arteries but was rapidly expressed in medial VSMCs by 1 day after injury (Figure 4B). This staining is distributed circumferentially around the vessel, and as with the aortic allografts, expression is almost exclusively limited to the medial VSMCs, because these cells also stain positively for SMC ξ-actin (Figures 4G and 4L). Intense AIF-1 expression was apparent in the medial VSMCs and neointimal cells 3 days after injury (Figures 4C, 4H, and 4M). The most striking observation at this time point is that AIF-1 was present in cells of the developing neointima, whereas these same cells were negative for SMC ξ-actin. Neointimal VSMCs that have switched from the contractile to the synthetic phenotype characteristically do not synthesize ξ-actin. AIF-1 expression appeared to decline by 14 days after balloon injury (Figures 4D and 4I). At this time point, a large neointima was evident; however, the most intense AIF-1 immunostaining localized to the medial VSMCs compared with neointimal cells. The neointimal cells that did stain appeared to localize to the luminal side of the injury (Figure 4I). This neointima is a mix of SMC ξ-actin–positive and –negative cells; however, not all SMC ξ-actin–positive cells stained positively for AIF-1 (Figure 4N). This indicates that AIF-1 expression is not limited to rejecting allografts but is also induced by mechanical injury of the coronary arteries as well. These results are also similar to our findings in balloon-injured rat carotid arteries with respect to the acute and transient kinetics of AIF-1 expression.

AIF-1 Protein Expression Is Induced by Cytokines in Human VSMCs

The lack of AIF-1 in uninjured vessels and its inducible expression in medial VSMCs after transplantation and mechanical injury suggested that expression of this protein in vivo is regulated by soluble factors. Because AIF-1 immunostaining localized to VSMCs, we examined induction of AIF-1 protein expression in primary cultured human coronary artery VSMCs stimulated with a variety of cytokines. In these experiments, cells were starved to quiescence by serum deprivation for 48 hours and exposed to 10% FCS, bFGF, IFN-γ, IL-1β, PDGF, TGF-β, and T-lymphocyte–conditioned media for 48 hours. Extracts were separated by SDS-PAGE, and AIF-1 was detected by Western analysis with specific antisera. The results of Figure 5 indicate that human AIF-1 is differentially induced by various soluble factors in cultured human VSMCs. Ten percent FCS, IFN-γ, and IL-1β are capable of inducing this protein, whereas AIF-1 is not expressed in unstimulated cells. bFGF, PDGF, and TGF-β can induce AIF-1 protein expression to a small degree. T-cell–conditioned media, which contains several soluble factors, elicited the strongest induction of AIF-1.

Because activated lymphocytes are among the first immune cells present in the arterial lesion and because T-lymphocyte–conditioned media elicited the strongest induction of AIF-1 protein in cultured VSMCs, we treated cultured human VSMCs with T-lymphocyte–conditioned media and examined extracts of these cells for AIF-1 protein by Western analysis at different time points. Figure 6 demonstrates that AIF-1 is not expressed in unstimulated cells but is induced by T-cell–conditioned media 24 hours after stimulation and peaks by 72 hours. These results indicate that in human VSMCs, AIF-1 is a cytokine-responsive protein and is particularly responsive to immune cell factors, suggesting that its induction in arteriopathic lesions is primarily dependent on inflammatory processes.

Overexpression of AIF-1 in Human VSMCs Increases Their Proliferative Capacity

The expression of AIF-1 in activated VSMCs led us to investigate whether expression of this protein was linked to cell growth. As an initial approach toward understanding the function of AIF-1 in VSMC activation, we determined the effects of overexpression of this protein on VSMC proliferation. Primary human VSMCs were transfected with either no plasmid (mock control), with pBK-CMV plasmid alone, or with pBK-CMV containing AIF-1 protein coding region.

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Human coronary artery VSMCs were transfected with either no plasmid (mock control), pBK-CMV plasmid alone, or pBK-CMV-AIF-1. Two days after transfection, cells were trypsinized, split 1:2, and left to grow in the presence of growth medium + G418 for 15 days, at which time the cells were then trypsinized and counted by using a standard hemocytometer. Data are from 3 independent experiments.
cDNA (pBK-CMV-AIF-1). Two days after transfection, cells were trypsinized and split 1:2, with one half left to grow in the presence of growth medium + G418 (Geneticin) for 15 days. The other half was saved for RNA isolation. After selection, the cells were trypsinized and counted. The results of 3 independent experiments are presented in the Table and demonstrate an average of 2.6 fold-increase in pBK-CMV-AIF-1–containing cells compared with pBK-CMV–containing cells. Because the transfection efficiency of primary human cells is low, it was necessary to use RT-PCR of RNA isolated from newly transfected cells to indicate that equal amounts of plasmid are present in control and in pBKCMV-AIF-1–transfected cells. Figure 7 indicates that these results are not due to differences in transfection efficiency, suggesting that human VSMCs that overexpress AIF-1 proliferate at a more rapid rate than do cells that do not.

**Discussion**

Two widely used and clinically relevant models of arterial injury are rat cardiac allograft rejection and swine balloon angioplasty. We initially focused on Lewis to Wistar-Furth allografts, which consistently develop an acute graft rejection within 12 days, and aortic tissue, which is almost exclusively composed of VSMCs. It has been shown that nonimmunosuppressed rat aortic allografts are not acutely rejected but develop arteriosclerotic alterations in the vascular wall that closely and histologically resemble the pathological changes observed in clinical transplants that undergo chronic rejection. These alterations include infiltration of mononuclear cells into the adventitia, endothelial cell hyperplasia, and SMC proliferation, resulting in thickening of the intima over time. Aortic allografts also resemble chronically rejecting human transplants biochemically with respect to plasma levels of thromboxane and prostacyclin. At the mRNA and protein level, AIF-1 is acutely and transiently expressed in aortic allografts (Figures 1 and 2). An induction of AIF-1 mRNA is detectable 24 hours after surgery, and AIF-1 protein is detectable shortly thereafter, at 3 days. mRNA and protein decline by 10 days after transplant. AIF-1 mRNA and protein are detectable at low levels in the Lewis to Lewis control animals, likely as a result of small numbers of macrophages and the moderate expression of growth factors that are found in isografted arteries.

Immunohistochemical analysis of donor aortas with AIF-1 antibody closely reflects the acute and transient expression pattern obtained by Western analysis and RT-PCR. It has been demonstrated that in aortic allograft rejection, IL-2 receptors (T lymphocytes) and OX42-positive cells (macrophages), although present in the adventitia, are generally absent in the media and intima until 20 days after transplant. Notwithstanding, it was possible that AIF-1 expression was due at least in part to infiltrating immune cells. Consequently, it was important to verify that the primary source of AIF-1 expression that we observed was derived from SMCs. Further, Figure 3 shows that AIF-1 is expressed in SMC α-actin–positive medial VSMCs. It is also interesting to note that in allografted rat aortas, medial thickening and cellular proliferation in medial and intimal VSMCs does not begin until 14 days after surgery. The early presence of mononuclear cells in the adventitia of the grafted aorta may be the source of cytokines, which activate the medial VSMCs, which in turn induce AIF-1 expression in these cells. Because AIF-1 expression is readily detectable in VSMCs by 3 days after transplantation, expression of this protein may be an early marker of graft rejection originating from the vessel itself.

At the cellular and molecular level, the vascular responses to mechanical and immune injury are similar. Immunohistochemical staining of donor aortas with AIF-1 antibody closely reflects the acute and transient expression pattern obtained by Western analysis and RT-PCR. It has been demonstrated that in aortic allograft rejection, IL-2 receptors (T lymphocytes) and OX42-positive cells (macrophages), although present in the adventitia, are generally absent in the media and intima until 20 days after transplant. Notwithstanding, it was possible that AIF-1 expression was due at least in part to infiltrating immune cells. Consequently, it was important to verify that the primary source of AIF-1 expression that we observed was derived from SMCs. Further, Figure 3 shows that AIF-1 is expressed in SMC α-actin–positive medial VSMCs. It is also interesting to note that in allografted rat aortas, medial thickening and cellular proliferation in medial and intimal VSMCs does not begin until 14 days after surgery. The early presence of mononuclear cells in the adventitia of the grafted aorta may be the source of cytokines, which activate the medial VSMCs, which in turn induce AIF-1 expression in these cells. Because AIF-1 expression is readily detectable in VSMCs by 3 days after transplantation, expression of this protein may be an early marker of graft rejection originating from the vessel itself.

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At the cellular and molecular level, the vascular responses to mechanical and immune injury are similar. Immunohistochemical staining of donor aortas with AIF-1 antibody closely reflects the acute and transient expression pattern obtained by Western analysis and RT-PCR. It has been demonstrated that in aortic allograft rejection, IL-2 receptors (T lymphocytes) and OX42-positive cells (macrophages), although present in the adventitia, are generally absent in the media and intima until 20 days after transplant. Notwithstanding, it was possible that AIF-1 expression was due at least in part to infiltrating immune cells. Consequently, it was important to verify that the primary source of AIF-1 expression that we observed was derived from SMCs. Further, Figure 3 shows that AIF-1 is expressed in SMC α-actin–positive medial VSMCs. It is also interesting to note that in allografted rat aortas, medial thickening and cellular proliferation in medial and intimal VSMCs does not begin until 14 days after surgery. The early presence of mononuclear cells in the adventitia of the grafted aorta may be the source of cytokines, which activate the medial VSMCs, which in turn induce AIF-1 expression in these cells. Because AIF-1 expression is readily detectable in VSMCs by 3 days after transplantation, expression of this protein may be an early marker of graft rejection originating from the vessel itself.
adventitia and circulation. This is in contrast to that observed for SMC α-actin–positive cells, which are more uniformly present throughout the neointima. Taken together, this infers that AIF-1 expression may be more of a marker of the initial VSMC response to inflammation rather than of the progression of intimal hyperplasia.

Immune cells secrete several soluble factors that stimulate VSMC migration, growth factor secretion, and cellular proliferation, and numerous studies have shown that VSMCs in allografted vessels and balloon angioplasty–injured arteries express several classes of cytokines and inflammatory factors.9,24 Inflammatory cytokines, such as IFN-γ and IL-1β, along with T-cell–conditioned media and FCS, are proliferative as well as inflammatory and display the most potent induction of AIF-1 compared with other factors.4,25 Although cytokines such as bFGF-1, PDGF, and TGF-β have been implicated as being responsible for the promotion of intimal hyperplasia in response to injury,1,4 these factors were capable of inducing AIF-1 protein expression only to a minor degree. Other studies have observed that in macrophages, AIF-1 mRNA expression is induced by T-cell factors, such as IFN-γ, and that this expression could be modulated by compounds that influence the inflammatory response.26 These studies have suggested that AIF-1 is not expressed in VSMCs; however, the VSMCs in those experiments were not cytokine-stimulated.13 Our results indicating that AIF-1 is not expressed in unstimulated VSMCs corroborate that earlier work. T-lymphocyte–conditioned medium contains several factors, including granulocyte-macrophage colony–stimulating factor, IFN-γ, IL-1, IL-6, and TNF-β, which are known to influence the VSMC phenotype, and it has been demonstrated that infiltration of the artery wall by T lymphocytes induces a change in VSMC phenotype, which is mediated by inflammatory, mitogenic, and antiproliferative factors.27,28 In agreement with what we observed with injured vessels in vivo, induction of AIF-1 occurs rapidly and is detectable at 24 hours of exposure to T-cell–conditioned medium (Figure 6).

Stimulation of cells with cytokines and mitogens results in a rapid transient increase in calcium levels,29 and mitogenic stimulation of arterial SMCs involves a flux of calcium ions through the plasma membrane.30,31 Calcium and its primary receptor protein, calmodulin, are required for cellular growth and survival, because both of these are essential for the entry of quiescent cells into the cell cycle in response to mitogenic signals.32,33 Not surprisingly, calcium channel blockers and calcium antagonists inhibit restenosis subsequent to mechanical (balloon angioplasty) and immunologic (transplant vasculopathy) insult.34–37 Overexpression of calmodulin in cultured cells enhances cell proliferation, primarily through a reduction in the length of the G1 phase of the cell cycle,8,9 and stable overexpression of several other calcium-binding proteins has also been shown to influence the cell cycle.12,13 With these observations as a starting point, we hypothesized a role for AIF-1 expression in VSMC growth.

Similar to other proteins that interact with calcium, AIF-1 is involved in cellular proliferation, because overexpression of this protein enhances the growth of human VSMCs. Stable transfection of AIF-1 cDNA into primary human VSMCs results in an average 2.6-fold increase in cell number (Table). Given the role of calcium regulation in growth control, one possible explanation for the growth-enhancing properties of AIF-1 is through its ability to interact with and possibly regulate the concentration of calcium ions in the cell.

The expression of AIF-1 in a localized cytokine-rich injury implies that AIF-1 may take part in inflammation-initiated activation events. The expression of AIF-1 in medial and intimal VSMCs before proliferation in vivo infers that this protein may function in the mediation of VSMC growth initiated by inflammatory stimuli. Additionally, although the vascular response to injury and development of restenosis has been shown to differ from species to species,28 the present study also indicates that AIF-1 expression is conserved, suggesting a fundamental function for this protein in vascular injury. In immune and mechanical vascular injury, immune cells play an important role in the initiation and progression of restenosis. However, the vascular pathobiology resulting in intimal hyperplasia is due to VSMC activation, making AIF-1 not only a surrogate marker of arterial injury but also a potential target of antirestenotic therapies.

Acknowledgments

The authors are grateful to Dr Howard Eisen for critical reading of the manuscript and Sheri Kelemen for technical advice.

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Expression of Allograft Inflammatory Factor-1 Is a Marker of Activated Human Vascular Smooth Muscle Cells and Arterial Injury
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Arterioscler Thromb Vasc Biol. 2000;20:1737-1744
do: 10.1161/01.ATV.20.7.1737

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