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The ability of AIF-1 to activate human vascular smooth muscle cells is lost by mutations in the EF-hand calcium-binding region

Michael V. Autieri*, Xing Chen

Department of Physiology, Cardiovascular Research Center, Temple University School of Medicine, Room 810, MRB 3420, N. Broad Street, Philadelphia, PA 19140, USA

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Abstract

Allograft Inflammatory Factor-1 (AIF-1) is a cytoplasmic calcium-binding protein expressed in vascular smooth muscle cells (VSMC) in response to injury or cytokine stimulation. AIF-1 contains a partially conserved EF-hand calcium-binding domain, and participates in VSMC activation by activation of Rac1 and induction of Granulocyte-Colony Stimulating Factor (G-CSF) expression; however, the mechanism whereby AIF-1 mediates these effects is presently uncharacterized. To determine if calcium binding plays a functional role in AIF-1 activity, a single site-specific mutation was made in the EF-hand calcium-binding domain to abrogate binding of calcium (AIF-1ΔA), which was confirmed by calcium overlay. Functionally, similar to wild-type AIF-1, AIF-1ΔA was able to polymerize F-actin in vitro. However, in contrast to wild-type AIF-1, over-expression of AIF-1ΔA was unable to increase migration or proliferation of primary human VSMC. Further, it was unable to activate Rac1, or induce G-CSF expression to the degree as wild-type AIF-1. Taken together, modification of the wild-type EF-hand domain and native calcium-binding activity results in a loss of AIF-1 function. We conclude that appropriate calcium-binding potential is critical in AIF-1-mediated effects on VSMC pathophysiology, and that AIF-1 activity is mediated by Rac1 activation and G-CSF expression.

Keywords: Allograft Inflammatory Factor-1; Calcium-binding domain; Vascular smooth muscle cell

Introduction

The neointima formation associated with vasculopathy subsequent to both immune and mechanical injury is a complex process involving several different cell types which secrete many different cytokines and growth factors seminal to the local inflammatory response [1]. Binding of these factors to their cognate receptors activates a calcium flux and series of protein kinases which in turn transmit the activation signal to the nucleus leading to transcription factor activation and gene expression [2]. Identification and functional characterization of genes expressed in response to vascular injury is a promising approach for the identification of targets to combat proliferative arteriopathy observed in transplant vasculopathy and restenotic subsequent to vascular interventional procedures [3].

Allograft inflammatory Factor-1 (AIF-1) is a 143 amino acid, calcium-binding protein. The human AIF-1 protein contains a 28 amino acid region corresponding to an EF-hand helix–loop–helix motif, a non-functional ancestral EF-hand motif, and consensus phosphorylation sequences for Casein Kinase II and Protein Kinase C (PKC) [4]. AIF-1 contains multiple PDZ domains which are important in mediating interactions for the assembly of large multi-protein complexes at specific subcellular locations [5]. AIF-1 has been implicated in the inflammatory process of several cell types, primarily macrophages and glial cells, where it is constitutively expressed [6]. AIF-1 is highly evolutionarily conserved, participating in the immune response in such phylogenetically distant species as Carp and marine sponges,

* Corresponding author. Fax: +1 215 707 5737.
E-mail address: mautieri@temple.edu (M.V. Autieri).

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suggesting a fundamental role in inflammatory processes [7,8]. We have focused on the role of AIF-1 in vascular smooth muscle cells (VSMC) activation and promotion of vascular proliferative diseases. AIF-1 is not detected in normal arteries, but is expressed in an acute and transient fashion in medial and neointimal VSMC in balloon angioplasty and allograft-injured arteries in several species, including humans [9–11]. Expression of AIF-1 in cardiac allografts correlates with the severity of rejection, and persistent expression is associated with development of clinical coronary artery vasculopathy (CAV) [10]. Correspondingly, AIF-1 is not expressed in unstimulated cultured human VSMC, but is strongly induced in response to inflammatory cytokines and T lymphocyte conditioned media [4,9]. We previously determined that the degree of proliferation of a rat SMC line is directly proportional to the amount of AIF-1 expressed [11]. This study also suggested that increased proliferation of these cells was due to a reduction in the length of the cell cycle. Over-expression of AIF-1 increased proliferation of primary VSMC by induction of granulocyte-colony stimulating factor (G-CSF) [12]. Neutralization of G-CSF reduced AIF-1-mediated proliferation, suggesting an autocrine growth loop. AIF-1 resides in the cytoplasm, but upon platelet-derived growth factor (PDGF) stimulation, it translocates to lamellipodia. AIF-1 binds to and polymerizes actin, and over-expression of AIF-1 in primary human VSMC leads to Rac1 activation and enhanced migration [13].

It is well established that stimulation of VSMC with cytokines and mitogens results in a rapid, transient increase in Ca^{2+} levels [14], and that proteins which buffer Ca^{2+} concentrations are involved in the regulation of signal transduction, transcription of immediate early genes, and cellular proliferation [15–17]. Although we have shown AIF-1 expression to have marked phenotypic effects on VSMC pathophysiology, the mechanisms whereby AIF-1 exerts its functional effects have not been explored. Our hypothesis is that calcium-binding regulates AIF-1 effects on VSMC activation. The purpose of this study is to determine if AIF-1 calcium binding plays a role in AIF-1-mediated VSMC activation. In this report, we show that a single amino acid substitution in the EF-hand calcium-binding domain eliminates the ability of AIF-1 to bind calcium. This elimination of Ca^{2+} binding has direct effects on AIF-1-mediated VSMC migration, VSMC proliferation, Rac1 activation, and G-CSF expression. This identifies important mechanistic insights by which AIF-1 expression may modulate VSMC pathophysiology and contribute to vascular proliferative diseases.

Materials and methods

Cells and culture

Human coronary VSMC were obtained as cryopreserved secondary culture from Cascade Corporation (Portland, OR) and subcultured in growth medium as described previously [13]. Cells from passages 3 to 6 were used in the described studies. The growth media were changed every other day until cells approached confluence. Platelet-Derived Growth Factor AB (PDGF), 20 ng/ml, was purchased from Sigma (St. Louis, MO).

Site-directed mutagenesis of AIF-1

The EF-hand region of the AIF-1 protein was mutated by a PCR-based strategy using the Quick-change mutagenesis kit from Stratagene (La Jolla, CA), according to the manufacturer's instructions. The change was designed to modify the conserved Ca^{2+} coordination positions in the EF-hand domain (residues 58–70 in AIF-1) [4,18–20]. The primers used were 5′ TAC ATG GAG TTT GCG GCA AAC TCC ATG TAT TTG 3′ which replaces aspartic acid with alanine at amino acid 58 to abrogate Ca^{2+} binding. To avoid cumbersome terminology, nonstandard abbreviations used are AIF-1ΔA for AIF-1D58A.

Viral construction and stable transduction

AIF-1 retrovirus (AIF-1RV) was constructed using a kit from Clontech (La Jolla, CA) as previously described [13]. Briefly, the protein coding region of the AIF-1 cDNA was inserted into the retroviral packaging vector pLXSN containing the gentimycin resistance gene. This was transfected into an ectotropic 293-derived packaging cell line [21]. Media were then collected from these cells and used to infect an amphitropic second packaging cell line, PT-67, according to the manufacturer's instructions. This supernatant containing recombinant high titer virus was then used to infect human VSMC in two 4-h exposures of viral supernatant in the presence of 8 μg/ml Polybrene (approximately 40–60% stable transduction was achieved) as described [13]. Stably transduced hVSMC G418 resistant cells were pooled from each transduction rather than individual clones to avoid the effects of clonal variation.

For proliferation assays, equal numbers of stable transfectants were seeded into 12-well plates at a density of 7500 cells/ml as described [12]. Medium was changed on the fourth day, and after 1, 4, and 7 days, cells were trypsinized and counted using a standard hemocytometer. Significance in differences between AIF-1 and mutants was analyzed by one-way ANOVA.

Calcium binding

The protein coding region of AIF-1 and AIF-1ΔA was cloned into the pGEX vector (Pharmacia), and bacterially produced AIF-1-GST fusion proteins were purified from lysates by glutathione-sepharose beads according to the manufacturer's protocols. AIF-1 protein was removed from GST by thrombin digestion and was subsequently used for