AIF-1 Expression Modulates Proliferation of Human Vascular Smooth Muscle Cells by Autocrine Expression of G-CSF

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Objective—Allograft inflammatory factor-1 (AIF-1) is associated with vascular smooth muscle cell (VSMC) activation and vascular injury. The purpose of this study was to characterize the molecular mechanism of AIF-1 growth-enhancing effects in human VSMC.

Methods and Results—Primary human VSMCs were stably transduced with AIF-1 retrovirus (RV). Impact on cell growth was evaluated by the increase in cell number, and the effects on gene expression were determined by cDNA microarray analysis. AIF-RV overexpressing cells grew significantly more rapidly than empty-RV control cells in growth medium and serum-reduced medium (P<0.01 and 0.02, respectively). cDNA microarray analysis and Western blotting on serum-starved AIF-1–transduced VSMCs identified increased mRNA expression of several cell cycle proteins and, surprisingly, the cytokine G-CSF. Addition of G-CSF caused a 75% increase in proliferation of VSMCs in the absence of serum growth factors. The proliferative effects of AIF-1 were abrogated by neutralizing antibodies to G-CSF (P<0.05), and AIF-1–transduced VSMCs are chemotactic for human monocytes. Increased expression of G-CSF and colocalization with AIF-1 positive cells were seen in diseased, not normal human coronary arteries.

Conclusions—This study indicates that AIF-1 enhances VSMC growth by autocrine production of G-CSF, and AIF-1 expression may influence VSMC–inflammatory cell communication. (Arterioscler Thromb Vasc Biol. 2004; 24:1217-1222.)

Key Words: allograft inflammatory factor-1 ■ G-CSF ■ proliferation

The origin of vascular proliferative syndromes as a consequence of interventional procedures or allograft rejection is inflammatory in nature. Injured endothelial cells secrete growth and chemotactic factors that recruit immune cells, which in turn secrete inflammatory cytokines. This localized production of cytokines elicits activation of normally quiescent medial vascular smooth muscle cells (VSMCs).1 Activated intimal VSMCs proliferate and synthesize cytokines, which they respond to in an autocrine fashion, contributing to a progressive loss of lumen diameter.2 Cross talk between vascular and immune cells through soluble factors also contributes to the dynamic and progressive nature of atherosclerosis and vascular restenosis. It is also established that activated immune cells and their soluble products influence VSMC phenotype and development of atherosclerosis.3,4 Because activated VSMCs are responsible for much of the obliterative thickening subsequent to arterial injury, functional characterization of genes involved in VSMC growth is important in identification of targets to combat proliferative arteriopathies.

Allograft inflammatory factor-1 (AIF-1) is a 143-aa, cytoplasmic, evolutionarily-conserved, calcium-binding protein.5 AIF-1 has been implicated in the inflammatory process of several cell types, primarily macrophages and glial cells, where it is constitutively expressed. Our work has focused on the role of AIF-1 in the vascular response to injury. AIF-1 is not present in normal arteries, but is expressed in an acute and transient fashion in medial and neointimal VSMCs in balloon angioplasty and allograft-injured arteries in several species, including humans.5,6,7 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).7 Similarly, AIF-1 is not expressed in unstimulated cultured human VSMCs, but is strongly induced in response to inflammatory cytokines and T lymphocyte conditioned medium.6 AIF-1 binds to and polymerizes actin, and overexpression of AIF-1 in primary human VSMCs leads to Rac1 activation and enhanced migration.8 Transfection of AIF-1 cDNA in a transformed rat SMC line is associated with increased growth of those cells.9 Although these studies suggest a close association with AIF-1 expression and VSMC pathophysiology, the mechanism whereby AIF-1 expression influences VSMC proliferation remains
largely unknown. In this article we explore the molecular mechanisms of AIF-1 expression on gene expression relating to proliferation of primary human VSMCs. The results presented show that even in serum-starved human VSMCs, AIF-1 overexpression results in increased proliferation and expression of the cytokine granulocyte-colony stimulating factor (G-CSF). AIF-1–induced VSMC proliferation is accompanied by cell cycle protein expression, and can be abrogated by G-CSF neutralization. AIF-1–expressing cells are also chemotactic for human monocytes. Immunohistochemical analysis of human coronary arteries with CAV demonstrate increased expression of G-CSF, which immunolocalizes with AIF-1 positive cells. We conclude that AIF-1–induced VSMC proliferation is an autocrine process mediated by G-CSF expression, and may represent a mechanism for progression of vascular proliferative diseases.

Materials and Methods

Cells and Culture

Human coronary VSMCs were obtained as cryopreserved secondary culture from Cascade Corporation (Portland, Ore) and subcultured as described. Cells from passages 3 to 6 were used in the described studies. G-CSF was purchased from Sigma. The THP-1 human monocyte cell line was obtained from the American Type Culture Collection and propagated in RPMI 1640 medium supplemented with 10% fetal calf serum.

Retroviral Construction and Stable Transduction

AIF-1 retrovirus (AIF-1-RV) was constructed using an kit from CLONTECH, according to manufacturer’s instructions as described. 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. Medium was changed on the fourth day, and after 1, 4, and 7 days cells were trypsinized and counted using a standard hemocytometer. Some assays used neutralizing G-CSF antibody or irrelevant antibody as negative control. To determine effects of G-CSF on VSMC growth, G-CSF at 1000, 100, or 10 pg/mL was added to VSMCs, either in growth medium or 0.5% FCS, and counted after 7 days. Neutralizing antibody to G-CSF (BD Biosciences) was added to cells at 2 μg/mL.

For migration assays, the bottom chamber of Transwell Boyden chamber plates (Costar) with 8-μm polycarbonate membrane pore size were seeded with stably-transduced VSMCs (50 000 cells per well). After cell adhesion, cells were washed with PBS, and growth medium was changed to medium containing 0.5% FCS. In some wells, neutralizing G-CSF antibody was added, and 48 hours later, 50 000 THP-1 monocytes in 0.5% FCS was added to the upper chamber, and cells were incubated for 8 hours at 37°C, after which cells were fixed and stained in Diff-Quick Cell Stain (American Hospital Supply). The upper layer was scraped free of cells, and cells that had migrated to the lower surface of the membrane were quantitated by counting 4 high-powered fields per membrane.

cDNA Microarray Analysis

Total RNA was isolated from 2 independently-derived groups of VSMCs stably transduced with empty vector or AIF-1-RV, which had been incubated in serum-reduced medium (0.5%) for 72 hours. Nylon cDNA microarrays specific for cell cycle or cytokine genes were purchased from SuperArray, Inc (Bethesda, Md), and RNA was labeled with 32P dCTP, hybridized, and washed according to manufacturer’s instructions. These arrays contain 96 probes specific for cell cycle or cytokine genes, including housekeeping genes. Changes in gene expression were detected by autoradiography and quantitated by scanning densitometry from film exposed in the linear range of detection. Signals were normalized to 3 different housekeeping genes included on the membrane.

Cytokine Assay, Western Blotting, and Tissue Processing and Immunohistochemistry

Cytokine assay, Western blotting, and tissue processing and immunohistochemistry were performed as described. For more detail, please see the online Methods section (available at http://atvb.ahajournals.org).

Results

AIF-1 Expression Enhances Growth of Human VSMC

Primary human coronary artery VSMCs were stably transduced with AIF-1-RV containing the protein coding region of AIF-1 cDNA (AIF-1-RV) or with vector alone (empty-RV), and stable transductants were selected with antibiotic. Individual colonies were combined to avoid the effects of clonal selection, and constitutive AIF-1 protein expression was verified by Western blot (not shown). Equal numbers of stable transductants were seeded into 12-well plates, and after 1, 4, and 7 days, cells were trypsinized and counted. Figure 1A demonstrates that primary human VSMCs that stably overexpress AIF-1 grow more rapidly than those that do not. A, Equal numbers of pooled human VSMCs stably-transduced with AIF-1-RV or empty vector were seeded into 12-well plates and grown in growth medium. B, VSMCs were seeded into 12-well plates in the presence of 0.5% FCS. After 1, 4, and 7 days, cells were trypsinized and counted in triplicate. Numbers on the y axis indicate cells per well. Shown are mean data from 4 independent transductions with similar results (P<0.01 for growth medium,** and P<0.02 for serum-reduced,* for 4 experiments).
cdk7, cyclin E, Skp1, and G-CSF mRNA and protein were noted in serum-starved VSMCs. Overall, differential expression of cdk6, cdk7, cyclin E, Skp1, and G-CSF was noted in AIF-1 versus control transductants. Particularly noteworthy were the increased levels of these proteins in AIF-1 transductants. Figure 2 shows that, in agreement with the cDNA microarray analysis in 3 independently-derived groups of pooled stable transductants, cdk 6, cdk 7, cyclin E, Skp1, and G-CSF were all increased in AIF-1 versus control transductants. This is representative of 3 similar experiments from 3 independently-transduced groups of VSMCs.

gate a direct effect of AIF-1 on cell growth in the absence of growth stimulatory factors, the previous experiment was duplicated, except that after 24 hours, cells were washed, growth medium was replaced with medium containing 0.5% FCS, and cells were counted at 1, 4, and 7 days. Figure 1B shows that AIF-1-transduced VSMCs can continue to proliferate in 0.5% FCS up to 7 days, whereas control empty vector VSMCs do not. The AIF-1-RV cells grow an average of 90% more rapidly than empty-RV cells. These data indicate that AIF-1 enhances serum-growth factor proliferation, and can also promote entry into the cell cycle in the absence of serum growth factors.

**AIF-1 Expression Enhances Expression of Cell Cycle Genes and G-CSF**

To delineate a mechanism for these differences in growth effects, we examined the transcriptional profile of cell cycle and cytokine genes by cDNA microarray analysis of 2 independently-derived pooled stable AIF-1 and empty vector transductants that had been serum-starved for 72 hours. As compared with empty vector controls, AIF-1-RV VSMCs show an increased level of genes that play a role in the progression of the earliest phases (G1, G1/S transition) of the cell cycle, including cyclin E, cdk6 6 and 7, and Skp1 (Figure I, available online at http://atvb.ahajournals.org). Differences in expression of genes that function in other parts of the cell cycle, such as proliferating cell nuclear antigen (PCNA) or cyclins A and B, were not noted. G-CSF was the only mRNA that was increased in both cytokine arrays. No differences in expression were noted for VSMC mitogens, including PCNA, basic fibroblast growth factor (bFGF), or transforming growth factor (TGF)-β.

Expression of these genes was further verified by Western analysis in 3 independently-derived groups of pooled stable transductants. Figure 2 shows that, in agreement with the cDNA microarrays, cdk 6, cdk 7, cyclin E, Skp1, and G-CSF were all increased in AIF-1 versus control transductants. Particularly noteworthy were the increased levels of these proteins in serum-starved VSMCs. Overall, differential expression of cdk6, cdk7, cyclin E, skp1, and G-CSF mRNA and protein were noted and verified in 5 independently-derived groups of pooled stable transductants. In all experiments, the expression of all of these proteins in AIF-1-transduced cells was at least 65% higher than empty vector cells (Table I, available online at http://atvb.ahajournals.org).

We next ascertained that G-CSF was secreted into the culture medium. Medium from equal numbers of AIF-1 and empty vector stable transductants were cultured in 0.5% FCS for 48 hours to minimize potential contribution by serum growth factors, at which time culture supernatant was assayed for G-CSF by ELISA assay (not shown). A significant difference was noted in that AIF-1 cells secreted G-CSF into culture medium in the 168 to 377 pg/mL range, whereas G-CSF was detected in supernatants from empty vector cells in the 26.9 to 76.2 pg/mL range (P<0.001 for 3 experiments). This indicates that the increases in G-CSF mRNA and protein noted in AIF-1–transduced cells were indeed being secreted into the culture supernatant by these cells.

**G-CSF Is Proliferative and Is Responsible for AIF-1-Induced VSMC Proliferation**

To determine whether G-CSF expression could impact VSMC proliferation, primary human VSMCs were incubated with varying concentrations of G-CSF and counted at 1, 4, and 7 days. At 7 days, significant differences in growth were noted at all G-CSF concentrations added to growth medium (P<0.05) (Figure 3). Perhaps more notably, both 10 and 100 pg/mL G-CSF can induce significant VSMC growth in serum-reduced medium (P<0.05 and 0.01, respectively). G-CSF at 1000 pg/mL had no statistically significant effect on VSMC growth. This study is the first to report that G-CSF has proliferative effects on human VSMC.

To more directly establish a relationship between AIF-1–induced G-CSF expression and AIF-1–induced VSMC proliferation, equal numbers of AIF-1 stable transductants were seeded into 12-well plates in the presence or absence of 2 to 8 μg/mL of neutralizing anti-G-CSF antibody. After 7 days, cells were counted. Figure 4 shows that addition of neutralizing anti-G-CSF antibody inhibited AIF-1–induced VSMC growth an average of 81% (P<0.05 for 3 experiments) in serum-reduced medium. Anti-G-CSF antibody had no significant effect on growth of empty vector cells. This result implicates G-CSF as an autocrine regulator of AIF-1–induced VSMC proliferation.

**AIF-1 Expression in VSMCs Enhances Monocyte Chemotaxis**

G-CSF is chemotactic for hematopoietic cells, and the effect of overexpression of AIF-1 in VSMC on migration of inflammatory cells was investigated. Equal numbers of AIF-1 and empty vector–transduced VSMCs were seeded into the lower chamber of a Boyden chamber. After attachment, cells were rinsed with PBS and incubated for 24 hours in serum-reduced (0.5% fetal calf serum) medium. Human monocytes were added to the upper chamber, and differences in chemotaxis between empty vector and AIF-1–transduced VSMCs were quantitated by counting migrated cells. Overall, a 325% increase in monocyte migration was noted in AIF-1–transduced human VSMCs, as compared with empty vector transduced cells (P<0.05 for 3 experiments) (Figure 5). No
significant difference in migration was found between empty vector–transduced VSMCs and monocytes cultured in the absence of chemokines, indicating that increased monocyte migration was caused by AIF-1 overexpression, not a suppression of migration in empty vector cells. Neutralizing antibody to G-CSF significantly reduced migration of monocytes an average of 73% (*P < 0.05 for 3 experiments). Neutralizing antibody to G-CSF was added to cells at 2 μg/mL. Medium and antibody were changed after 4 days, and cells were counted in triplicate after 7 days. Significant inhibition of growth in AIF-1–transduced cells was noted in the presence of anti–G-CSF antibody (*P < 0.05 for 3 experiments using 3 independently derived groups of stably-transduced VSMCs). * No inhibition was observed using control antibody. Numbers on the y axis indicate cells per well. Shown is a representative experiment.

G-CSF Is Upregulated in Injured Human Coronary Arteries

It was important to determine the pathophysiological relevance of G-CSF expression in VSMCs. Figure 6 illustrates that G-CSF is not expressed in a normal human coronary artery, but is detectable in neointimal cells in a coronary artery from a patient with severe transplant arteriopathy, thus associating increased G-CSF expression with arterial injury. Immunohistochemical analysis of serial sections from vessels with CAV demonstrate G-CSF colocalization with AIF-1 in neointimal cells (Figure 6C through 6E).

Discussion

Studies from several varied systems demonstrate AIF-1 expression in infiltrating macrophages in rat cardiac allografts, proliferating microglial cells in response to injury, activated murine T helper cells, and the allograft response in sponges. This evolutionarily conserved expression pattern implicates an important function for AIF-1 in inflammatory cells, but little is known about the functional ramifications of AIF-1 expression, particularly in vascular cells. We have previously determined that AIF-1 transfection can influence proliferation of a transformed rat SMC line. AIF-1 is also strongly expressed in myelofibroid-appearing cells in neointima of human coronary arteries with CAV, which are generally proliferative in nature. Primary human coronary artery VSMCs, although adapted to culture conditions, are a good model in which to study cell proliferation, in that they can be quiesced by serum deprivation and can readily re-enter the cell cycle on growth factor stimulation.

The observation that AIF-1 could promote VSMC proliferation in the absence of serum growth factors implies that AIF-1 can advance entry into the cell cycle. cDNA microarrays were then used as an initial screen of serum-starved, stably-transduced cells to ascertain a mechanism for these growth effects. In 2 independently-derived groups of cells, AIF-1–transduced VSMCs expressed more cyclin E, cdk6, cdk7, and Skp1 mRNA than control cells. This increased expression was confirmed at the protein level in 3 additional groups of independently-derived cells by Western blot.

Each of these proteins are specifically involved in the earliest phases of the cell cycle. Cdk6 becomes activated in mid-G1 in response to growth factors, where it forms a complex with cyclin D, linking growth factor stimulation with the onset of cell cycle progression. The activated cdk6/cyclin D complex subsequently leads to activation of the cdk2/cyclin E complex at the G1/S phase transition. The CDK-activating kinase complex consists of cdk7 and cyclin H, which then phosphorylates other cdk2 and cdc2. Skp1 binds the cdk2/cyclin A complex and regulates the cell cycle by the timely destruction of numerous regulatory proteins such as cyclins. The observation that AIF-1–transduced
VSMCs continue to proliferate in low serum is likely a result of aberrant expression of G₁-specific cell cycle proteins.

Entry into the cell cycle is driven by growth factors, and we considered whether the cell cycle protein expression in AIF-1 VSMCs was a consequence of cytokine expression. cdNA microarray analysis and Western blotting of serum-starved cells determined that G-CSF was also increased in AIF-1 transductants. Expression of this cytokine was unexpected, as G-CSF is a lineage-restricted hematopoietic growth factor that stimulates terminal mitotic divisions and the final cellular maturation of hematopoietic progenitors, particularly granulocytes. One study reports that interleukin (IL)-1β stimulated release of G-CSF by human VSMCs, but its effects on VSMC proliferation have not been reported. The ED₅₀ for mouse myeloblastic NFS-60 cell proliferation is 100 pg/mL, and AIF-1–expressing cells secrete G-CSF into serum-reduced culture medium in the 168 to 377 pg/mL range. In a myeloid cell line, G-CSF induces Egr-1 upregulation through interaction of serum response element–binding proteins. Because Egr-1 plays an essential role in vascular cell growth and pathophysiology, we hypothesized that G-CSF would also promote VSMC proliferation. We found that G-CSF in the 10 to 100 pg/mL range can promote VSMC proliferation regardless of the absence of serum growth factors. It was unexpected that 1000 pM did not significantly increase growth above 100 pM levels, but is similar to the effect observed on human endothelial cells in which G-CSF–induced thymidine incorporation peaked at 50 pM, and was significantly lower at 500 pM. Although known for some time to be proliferative for granulocytes, this is the first study to report that G-CSF has growth-enhancing effects on human VSMC, and confirms that this cytokine may exert pleiotrophic effects outside the hematopoietic system.

Neutralizing anti–G-CSF antibodies were used to inhibit AIF-1–induced VSMC growth in serum-reduced medium. This directly links AIF-1–induced VSMC growth with AIF-1–induced G-CSF production, leading us to conclude that AIF-1 expression induces autocrine growth regulation of human VSMC by endogenous production of G-CSF. Other investigators have found that overexpression of AIF-1 leads to induction of cytokine expression. Watano and colleagues found that transfection of AIF-1 into a mouse macrophage cell line results in production of IL-6, -10, and -12 in response to lipopolysaccharide stimulation. None of these cytokines, nor potent VSMC mitogens such as platelet derived growth factor or bFGF were differentially expressed in unstimulated AIF-1 versus empty vector VSMC on cdNA arrays.

G-CSF is proliferative and chemotactic for hematopoietic cells. Similarly, significant differences were noted between AIF-1 and empty vector–transduced VSMCs in their ability to stimulate migration of human monocytes. Neutralizing antibody to G-CSF significantly reduced, but could not completely abrogate, this migration, demonstrating that AIF-1–mediated production of G-CSF was responsible, at least in part, for monocyte migration. Immunohistochemical analysis of injured human coronary artery demonstrates G-CSF colocalization with AIF-1 in SMC α actin-positive neointimal cells. Together, this points to an important role for AIF-1, not only in autocrine growth of VSMC, but in cytokine production and the potential for paracrine cross talk between activated VSMC and infiltrating leukocytes leading to progression of the vascular lesion.

We have previously reported that even in serum-starved VSMCs, expression of AIF-1 in primary human VSMC leads to
increased migration and activation of the Ras family member, Rac1, the activity of which regulates cell migration.8 In VSMCs, mechanisms involved in cell cycle progression are also involved in cell migration, and these processes are mediated, at least in part, by CDK expression and activity.23 Further, VSMCs migrate in the mid to late G1 phase compared with cells in later phases that are unable to migrate,24 which is consistent with our findings that expression of both CDK 6 and CDK 7 are upregulated in AIF1–transduced cells. In bovine airway SMC, Rac1 activation has been shown to induce transcription of the cyclin D1 promoter, suggesting that Rac1 is a proximal activator of cyclin D1 transcription.25 In our experiments, overexpression of cyclin D1 promoter, suggesting that Rac1 is a proximal activator of cyclin D1 transcription.25 In our experiments, overexpression of AIF-1 increased cyclin E but not cyclin D1 expression. This may be reflective of species differences or distinction between origin of the SMC.

No studies directly linking Rac1 and G-CSF expression have been reported in VSMCs. In granulocytes, Rac1 can be activated by G-CSF, suggesting the endogenously produced G-CSF in AIF1–expressing cells may activate Rac1 and lead to both enhanced migration and proliferation.26 Conversely, the G-CSF promoter contains elements for transcription factors activated by kinases downstream from Rac1, including NF-kB, CREB, and API, among others, allowing for the possibility that Rac1 signaling is responsible for transcription of G-CSF. Consequently, future studies will determine whether the endogenously produced G-CSF in AIF1–expressing cells is responsible for AIF-1 effects on Rac1 activation, or whether AIF-1 activation of Rac1 is responsible for transcription of G-CSF.

In summary, this study shows that in the absence of serum growth factors, expression of AIF-1 in human VSMCs leads to their proliferation and expression of cell cycle proteins. Second, G-CSF is also expressed in and secreted by these cells, the neutralization of which abrogates AIF1–induced VSMC proliferation. Third, AIF1–transduced cells are chemoattractant for human monocytes, which is also abrogated by G-CSF neutralization. Finally, G-CSF expression is increased in injured human coronary arteries. Based on these data, we propose a scenario in which AIF1 initiates Rac1 activation and expression of G-CSF, leading to expression of key cell cycle proteins, thus allowing proliferation of VSMCs. Our working hypothesis is that AIF-1 expression contributes to the development of intimal hyperplasia by enhancing migration and proliferation of activated VSMCs. AIF1–induced expression of G-CSF also has implications for vascular-inflammatory cell communication.

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Figure I: Bar chart showing percent increase over empty vector for different proteins: cdk 6, cdk 7, cyclin E, skp 1, and G-CSF.