



# Inhibition of allograft inflammatory factor-1 expression reduces development of neointimal hyperplasia and p38 kinase activity

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## KEYWORDS

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Smooth muscle cell;  
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p38;  
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Migration

**Aims** Allograft inflammatory factor-1 (AIF-1) is a calcium-binding, scaffold-signalling protein expressed in vascular smooth muscle cells (VSMCs) in response to injury. The effects of AIF-1 attenuation on development of intimal hyperplasia are unknown, and the molecular mechanisms of these effects remain uncharacterized. The goals of the present study were to determine whether AIF-1 knockdown reduced VSMC proliferation, migration, and intimal hyperplasia, and determine AIF-1 effects on signal transduction in VSMCs.

**Methods and results** Balloon angioplasty-injured rat carotid arteries transduced with adenovirus to overexpress AIF-1 (AdAIF-1) significantly increased, and adenovirus to knock down AIF-1 (AdsiRNA) expression significantly decreased neointimal formation compared with green fluorescent protein (AdGFP) and Adscrambled controls ( $P < 0.05$  and  $P < 0.01$ ,  $n = 6$ ). Primary rat VSMCs transduced with AdAIF-1 displayed a significant increase in proliferation, and AdsiRNA-transduced VSMCs proliferated significantly more slowly than controls ( $P < 0.05$ ). VSMCs transduced with AdAIF-1 show increased migration when compared with control VSMCs ( $P < 0.01$ ). Rat VSMCs transduced with AdAIF-1 showed constitutive and prolonged activation of the mitogen-activated protein kinase p38, whereas AdsiRNA-treated VSMCs showed decreased p38 activation compared with AdGFP ( $P < 0.05$ ). Immunohistochemical analysis of AdAIF-1-transduced carotid arteries showed increased staining with a phospho-specific p38 antibody compared with AdGFP-transduced arteries. A specific p38 inhibitor abrogated AIF-1-induced VSMC proliferation, but not AIF-1-induced migration.

**Conclusion** Taken together, AIF-1 expression plays a key role in the development of neointimal hyperplasia. AIF-1 expression enhances the activation of p38 MAP kinase. AIF-1-enhanced proliferation is p38 kinase dependent, but AIF-1-enhanced VSMC migration is p38 independent.

## 1. Introduction

In both mechanical and immune-mediated arterial injury, the cytokine-induced activation and proliferation of medial vascular smooth muscle cells (VSMCs) is one of the most critical cellular events in the formation of neointimal hyperplasia. This is characterized by a localized response to injury in which cytokines and growth factors activate a series of protein kinase-mediated signal transduction pathways, resulting in gene expression and modulation of VSMC phenotype.<sup>1</sup> Several studies have shown that several signal transduction proteins, including p44/42, extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), and Jun N-terminal kinase (JNK),

are activated after arterial injury.<sup>2–5</sup> Selective inhibition of several protein kinases have an inhibitory effect on VSMC activation and development of neointimal formation after balloon injury.<sup>6,7</sup> Characterization of injury-responsive proteins that regulate signal transduction pathways represent targets for therapeutic approaches for several vascular pathologies including atherosclerosis, restenosis, and allograft vasculopathy.

Allograft inflammatory factor-1 (AIF-1) is a 143 amino acid, cytoplasmic, calcium-binding protein. AIF-1 is constitutively expressed in inflammatory tissue and glial cells, and has been implicated in the inflammatory process of several cell types, primarily macrophages and glial cells. AIF-1 is evolutionarily conserved and involved in inflammation in such phylogenetically distant species as Carp and marine sponges.<sup>8,9</sup> In humans, the AIF-1 gene maps to the major histocompatibility complex class III region on

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chromosome 6p21.3, which is densely clustered with genes involved in the inflammatory response, including TNF $\alpha$  and  $\beta$ , and NF- $\kappa$ B.<sup>10</sup>

We have determined an important role for AIF-1 in VSMC pathophysiology. Although constitutively expressed in lymphoid tissue, AIF-1 is not expressed in unstimulated VSMCs, but is rapidly induced *in vivo* in response to mechanical and allograft injury, and in cultured VSMCs by inflammatory cytokines.<sup>11</sup> Persistent expression of AIF-1 in cardiac allografts is predictive of development of clinical transplant vasculopathy.<sup>12</sup> AIF-1 has molecular signatures of a scaffold-signalling protein, including several PDZ interaction domains, which are important in mediating interactions of multi-protein complexes.<sup>13</sup> In unstimulated VSMCs, AIF-1 resides in the cytoplasm anchored to actin, but translocates to leading edge lamellipodia in stimulated VSMCs.<sup>14</sup> Overexpression of AIF-1 in VSMCs results in increased cell cycle protein expression and activation of the small GTPases Rac1 and Rac2.<sup>14–16</sup>

Our previous study has shown that chronic overexpression of AIF-1 in transgenic mice increases intimal hyperplasia in response to ligation injury, with subsequent increases in PAK1 and p38 phosphorylation in VSMCs.<sup>17</sup> While an informative study, the effects of AIF-1 abrogation on development of intimal hyperplasia have not been investigated, nor has the molecular pathway(s) responsible for AIF-1 enhancement of migration and proliferation in VSMCs been characterized. AIF-1 knockout mice are not available. Consequently, to test the hypothesis that the abrogation of AIF-1 expression ameliorates the development of intimal hyperplasia in response to injury, it is necessary to use the rat carotid balloon angioplasty model and modify AIF-1 expression by adenovirus. The results of the present study indicate a direct relationship between AIF-1 expression and neointimal formation *in vivo*. This study also implies that there are dual pathways of AIF-1 activity, a p38 MAPK-dependent pathway leading to AIF-1-enhanced VSMC proliferation, and a p38 MAPK-independent pathway leading to AIF-1-enhanced VSMC migration. These results suggest that AIF-1 expression is an important step in the development of intimal hyperplasia subsequent to vascular injury.

## 2. Methods

### 2.1 Adenovirus construction

Recombinant adenoviral vectors containing HA-tagged AIF-1 coding region (AdAIF-1) cloned into the pShuttle vector were prepared using the AdEasy system as described previously.<sup>18</sup> The AIF-1 siRNA construct (AdsiRNA) previously described was cloned into the siRNA expression vector, pRNA-U6.1/shuttle, and was prepared in an identical way.<sup>19</sup> Adenoviral green fluorescent protein (AdGFP) expressed in the same viral vector was used as control. Scrambled siRNA adenovirus was purchased from Vector Biolabs (Philadelphia, PA, USA).

### 2.2 Rat left common carotid artery balloon angioplasty and gene transfer

Left common carotid artery balloon angioplasty was performed on 350 g male Sprague–Dawley rats (Charles River Breeding Laboratory Inc., Wilmington, MA, USA), as we described previously.<sup>20</sup> After balloon injury, a cannula was introduced into the common carotid artery and the distal injured arterial segment isolated by temporary clips placed midway in the injured segment and at the orifice of the

internal carotid artery. This space was filled with either adenoviral GFP, AIF-1HA, scrambled sequence, or AIF-1siRNA (final titer =  $1.25 \times 10^9$  pfu /mL). Incubation was allowed to proceed for 15 min and then the solution was retrieved, the cannula removed, blood circulation restored, and the wound closed. The vessels were harvested 15 days later, fixed, and histology was determined as described previously.<sup>11</sup> This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and Temple University.

### 2.3 Cells, culture, migration, and proliferation assays

Primary rat VSMCs were obtained as cell explants from aorta of Sprague–Dawley rats and maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (FBS) as described previously.<sup>15</sup> Cells were positive for smooth muscle cell actin, and passage 3–5 were used. *In vitro* gene transfer into VSMCs was performed by incubation with 40 MOI either adenoviral GFP, AIF-1, or AIF-1siRNA for 2 h at 37°C. Twenty-four hours after infection, cells were used for proliferation, or 48 h for migration. This time was chosen because it took at least 24 h for appreciable adenoviral protein expression to be detected, and stability and consistency of expression of the AdsiRNA was most consistent 48 h post-infection (see Supplementary material online, Figure S1). For proliferation, equal numbers of stable transfectants were seeded into 24-well plates at a density of 4000 cells/mL as described previously.<sup>15,16</sup> Growth medium was changed on the fourth day, and after 4 and 7 days, cells were counted by using a standard haemocytometer. SB202190 was purchased from EMD and used at 10  $\mu$ M. Migration was performed in standard Boyden Chamber migration assays as we have described previously.<sup>14</sup> Experiments were performed in triplicate from three independently transduced groups of VSMCs. Scratch wounding was performed as we previously described, and wound area was quantified by image analysis.<sup>14</sup>

### 2.4 Immunohistochemistry and morphometry

Five micrometre sections from injured rat arterial tissue fixed in methanol were deparaffinized and blocked in 10% goat serum, incubated with primary antibody; anti-AIF-1, phospho-, and total p38 (Cell Signalling, Inc.), at 2  $\mu$ g/mL in 1% BSA/PBS and were applied for 1 h, followed by biotinylated secondary antibody (1:200), followed by avidin-biotin peroxidase complex each for 30 min. Non-specific isotype antibodies were used as negative controls. Staining was visualized with the substrate diaminobenzidine (Vector) producing a reddish-brown colour and counterstained with haematoxylin as described previously.<sup>12</sup> Digitized images were measured and averaged from at least three representative 5  $\mu$ m-thick stained tissue sections at least 75–100  $\mu$ m apart per carotid artery using Image Pro Plus (Media Cybernetics). Seven animals per condition were used. The circumference of the lumen, the area encircled internal elastic lamina (IEL), and the external elastic lamina (EEL) were quantified. The medial area was calculated by subtracting the area defined by the IEL from the area defined by the EEL, and intimal area calculated as the difference between the area inside the IEL and the luminal area using an automated computer-based image analyzer (Image-Pro<sup>®</sup> Plus).

### 2.5 Western blotting

Vascular smooth muscle cell extracts were prepared as described previously.<sup>15</sup> Membranes were incubated with a 1:2000 dilution of primary antibody, and a 1:2000 dilution of secondary antibody. Monoclonal anti-GAPDH was from Biogenesis, Inc. Equal protein concentrations of cell extracts were determined by Bradford assay and equal loading on gels were verified by Ponceau S staining of the membrane. Quantification of phosphorylation was normalized to

levels of total p38 protein. Reactive proteins were visualized using enhanced chemiluminescence (Amersham).

## 2.6 Statistical analysis

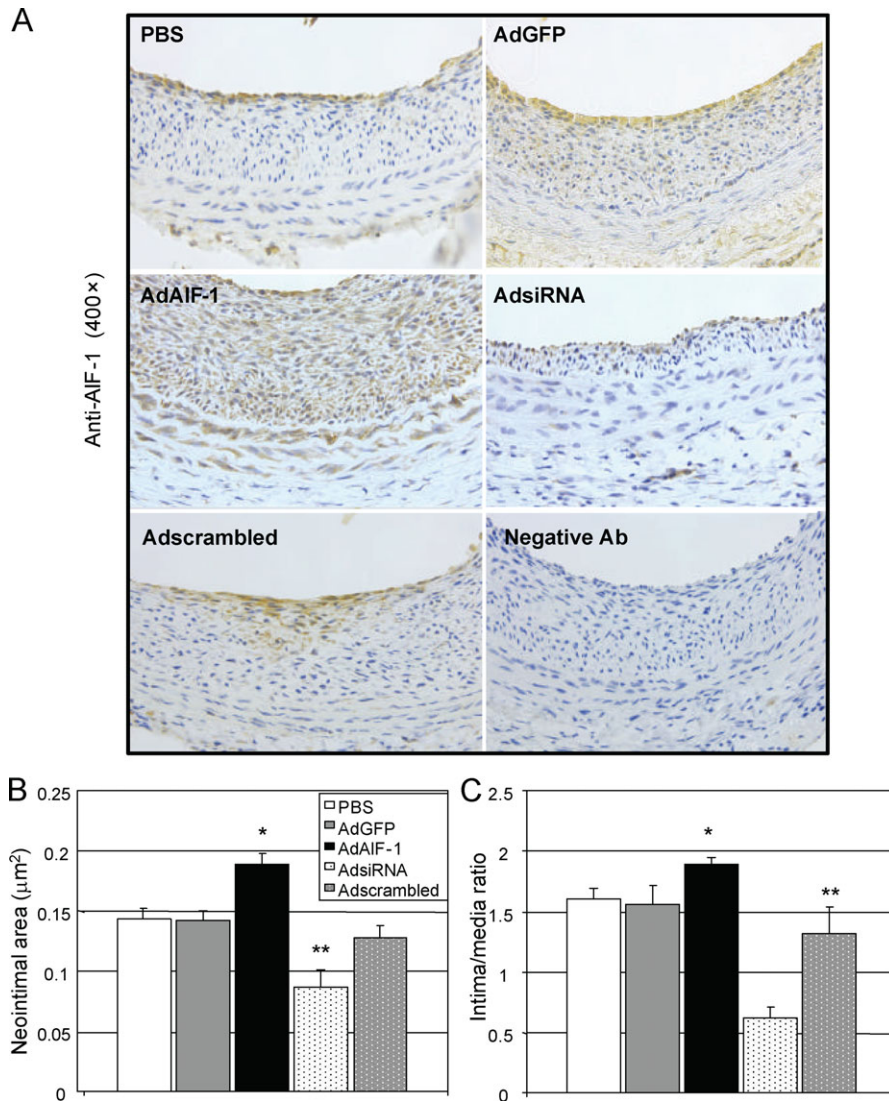
Results are expressed as mean  $\pm$  SE. Differences between groups were evaluated with the use of ANOVA, with the Newman-Keuls method applied to evaluate differences between individual mean values and by paired *t* tests where appropriate, respectively. Differences were considered significant at a level of  $P < 0.05$ .

## 3. Results

### 3.1 Allograft inflammatory factor-1 expression mediates development of neointimal hyperplasia

Allograft inflammatory factor-1 is not expressed in uninjured arteries, but is rapidly expressed in medial and neointimal VSMCs in response to injury.<sup>11</sup> Currently, nothing is known about the effects of AIF-1 knockdown on development of

intimal hyperplasia. As AIF-1 knockout mice are unavailable, it was necessary to utilize the rat carotid artery angioplasty injury model and adenoviral gene transfer. In these experiments, rat carotid arteries were balloon-injured, then infected with recombinant adenovirus encoding AIF-1 protein (AdAIF-1), green fluorescent protein control (AdGFP), or vehicle (PBS) only. An AIF-1 siRNA construct which has been shown to inhibit AIF-1 expression in murine macrophages was also delivered by adenovirus (AdsiRNA), as well as an siRNA scrambled control (Adscrambled) adenovirus.<sup>19</sup> Fifteen days post-adenovirus infection and injury, neointimal hyperplasia was assessed by morphometry and quantified. *Figure 1* shows that neointimal area in balloon-injured carotid arteries transduced with AdAIF-1 was significantly increased compared with AdGFP and vehicle alone ( $0.177 \pm .009$  vs.  $0.143 \pm 0.007$  and  $0.144 \pm 0.008 \mu\text{m}^2$ , for AdGFP and vehicle alone, respectively,  $P < 0.05$ ). Moreover, AdsiRNA transduction significantly decreased neointimal formation compared with AdGFP and with vehicle



**Figure 1** Modulation of allograft inflammatory factor-1 (AIF-1) expression influences neointimal hyperplasia. (A) Adenoviral delivery to balloon angioplasty-injured rat carotid arteries, harvested 15 days post-injury. Representative sections from seven animals,  $\times 40$  magnification. Sections stained with anti-AIF-1 antibody,  $\times 400$  magnification. Red-brown indicates positive immunoreactivity. Morphological and statistical analysis of effect of modulation of AIF-1 expression on neointima formation. (B) Neointimal area. (C) Intima/media ratio. Data shown are means  $\pm$  SEM. Significance vs. AdGFP determined by ANOVA ( $n = 7$ ). \* $P < 0.05$ , \*\* $P < 0.01$ .

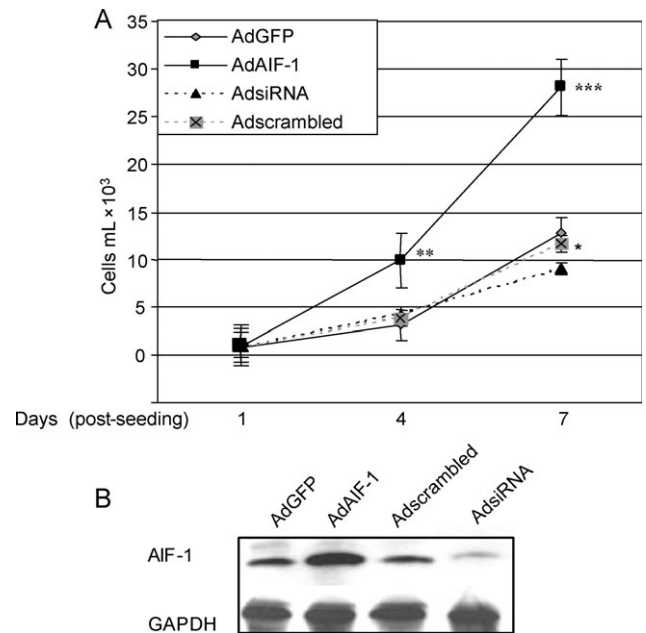
alone ( $0.087 \pm .014$  vs.  $0.143 \pm .007$  and  $0.144 \pm .008 \mu\text{m}^2$ , for AdGFP and vehicle alone, respectively,  $P < 0.01$ ). Scrambled siRNA showed no difference from AdGFP or vehicle controls. These differences are reflected in the intima/media ratio, in which significant differences are noted between AdAIF-1 and AdsiRNA vs. vehicle and AdGFP ( $1.98 \pm 0.06$ ,  $0.6 \pm .09$ ,  $1.6 \pm 0.1$ ,  $1.56 \pm 0.15$ , respectively,  $P < 0.05$  for AdAIF-1 and  $P < 0.01$  for AdsiRNA, vs. controls). Balloon-injured arteries in which phosphate-buffered saline (vehicle) showed an identical response as AdGFP arteries. Because AIF-1 is constitutively expressed in inflammatory cells, it was important to use immunohistochemistry to discern AIF-1 expression in medial VSMCs and neointimal cells. AIF-1 expression was verified by immunohistochemistry of arteries using anti-AIF-1 antibody (Figure 1A). AdAIF-1-transduced arteries demonstrate increased expression of AIF-1 in both neointimal and medial VSMCs, compared with AdGFP-transduced arteries. AdsiRNA-transduced arteries do show some AIF-1 immunoreactivity in the neointima. However, they demonstrate decreased expression of AIF-1, particularly in medial VSMCs, when compared with AdGFP-transduced arteries. No difference between groups was noted in medial thickness. Taken together, these data indicate that AIF-1 expression is tightly associated with the development of neointimal hyperplasia.

### 3.2 Allograft inflammatory factor-1 expression regulates vascular smooth muscle cell proliferation

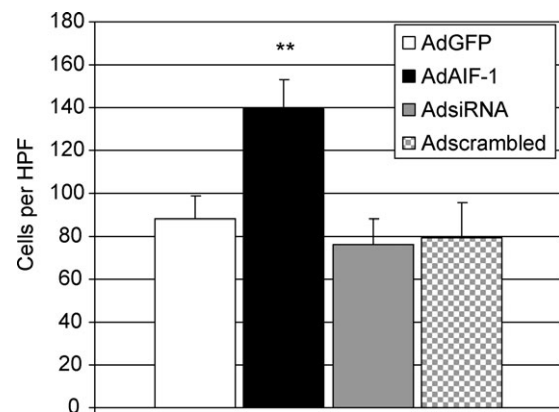
Uncontrolled proliferation of VSMCs in response to injury contributes to luminal narrowing.<sup>1</sup> To determine a cellular basis for AIF-1 modulation of neointimal hyperplasia, primary rat VSMCs were transduced with 40 MOI of AdGFP, AdAIF-1, or AdsiRNA. Twenty four hours later, equal numbers were seeded into 24-well trays and counted at 1, 4, and 7 days after seeding. Figure 2 shows that overexpression of AIF-1 results in a significant increase in cell number after 7 days ( $28.1 \times 10^3 \pm 27.8 \times 10^2$  vs.  $12.7 \times 10^3 \pm 15.9 \times 10^2$  for AdAIF-1 and AdGFP, respectively,  $P < 0.001$ ). In contrast, AdsiRNA-expressing cells grew significantly more slowly than control ( $8.8 \times 10^3 \pm 6.7 \times 10^2$  vs.  $12.7 \times 10^3 \pm 15.9 \times 10^2$  for AdsiRNA and AdGFP, respectively,  $P < 0.05$ ). Scrambled siRNA showed no difference from controls. To confirm that these growth differences correlate with AIF-1 expression, western blot of lysates from these cells demonstrated changes in AIF-1 protein levels (Figure 2B). The significant differences in cell numbers noted between AIF-1-overexpressing and AIF-1-underexpressing VSMCs compared with control cells suggests a strong association between AIF-1 expression and VSMC proliferation.

### 3.3 Allograft inflammatory factor-1 expression regulates vascular smooth muscle cell migration

To determine effects of AIF-1 expression on regulation of migration, rat VSMCs transduced with 40 MOI of AdGFP, AdAIF-1, or AdsiRNA in 0.5% FBS were seeded into the top chamber of Boyden chambers. Platelet-derived growth factor (PDGF) was added to the bottom chamber, and 3 h later migrated cells were counted. Figure 3 shows that rat VSMCs transduced with adenoviral AIF-1 migrate significantly more rapidly compared with controls ( $139.1 \pm 14.4$  vs.  $88.4 \pm 10.1$  cells/HPF for AdAIF-1 vs. AdGFP,  $P < 0.01$ ).



**Figure 2** Modulation of allograft inflammatory factor-1 (AIF-1) expression influences proliferation of rat VSMCs. (A) Primary rat VSMCs were transduced with 40 MOI of AdGFP, AdAIF-1, Adscrambled, or AdsiRNA and counted after seeding. Shown are means from three independent transductions with similar results. Asterisk indicates significant difference from AdGFP control ( $P < 0.05$ ). (B) Rat VSMCs infected with adenovirus as described in 'A' were stimulated with 10% FBS for 72 h to induce endogenous AIF-1 expression and immunoblotted with AIF-1 or GAPDH antibody.

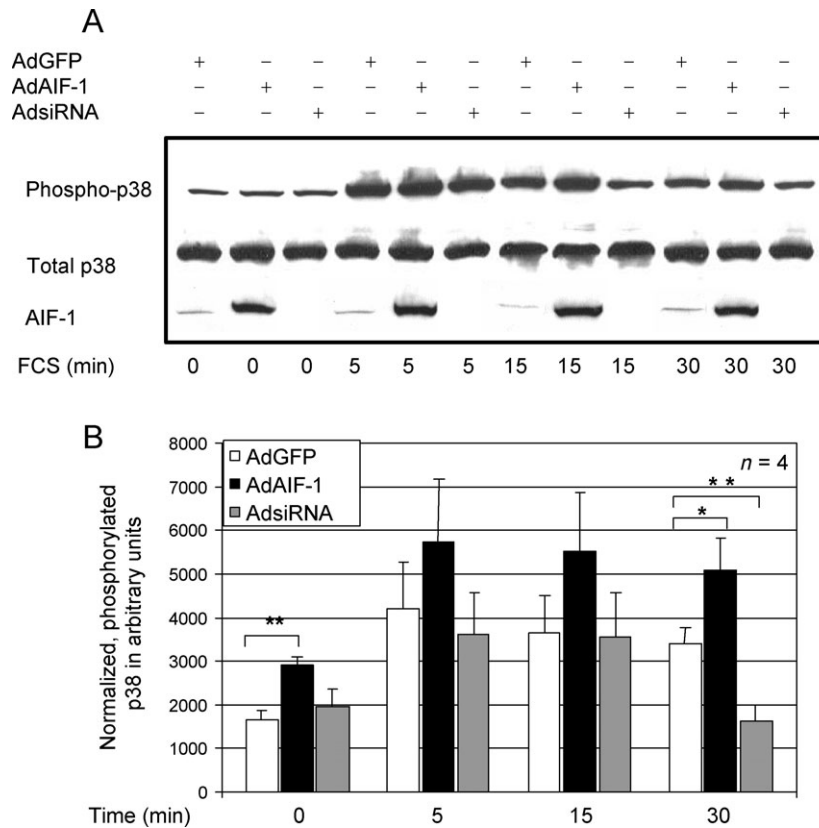


**Figure 3** Modulation of AIF-1 expression influences migration of Rat VSMCs. Primary rat treated as in Figure 2. Forty-eight hours later, VSMCs were seeded onto Boyden chamber membranes and exposed to 20 ng/mL PDGF for 3 h. Values are means from three experiments performed in triplicate from three independently transduced groups of VSMCs. Asterisk indicates significant difference from AdGFP control ( $P < 0.01$ ).

There was no significant difference in VSMCs transduced with AdsiRNA vs. AdGFP or Adscrambled transduced VSMCs ( $76.3 \pm 12.1$  vs.  $88.4 \pm 10.1$  cells/HPF for AdsiRNA and AdGFP, respectively). These data indicate that while overexpression contributes to enhanced VSMC migration, reduction of AIF-1 expression does not diminish it.

### 3.4 Allograft inflammatory factor-1 expression regulates p38 activation

Vascular smooth muscle cell activation in response to injury and serum growth factors is mediated by the coordinated



**Figure 4** Modulation of AIF-1 expression alters p38 protein kinase phosphorylation in VSMCs. Cell lysates were immunoblotted with antibodies for the phosphorylated form of p38 MAPK. (A) Representative immunoblot of VSMCs treated as described. (B) Combined statistical analysis. Values and means from four experiments using independently transduced groups of VSMCs. Asterisk indicates significant difference from AdGFP control ( $P < 0.05$  or  $0.01$ ).

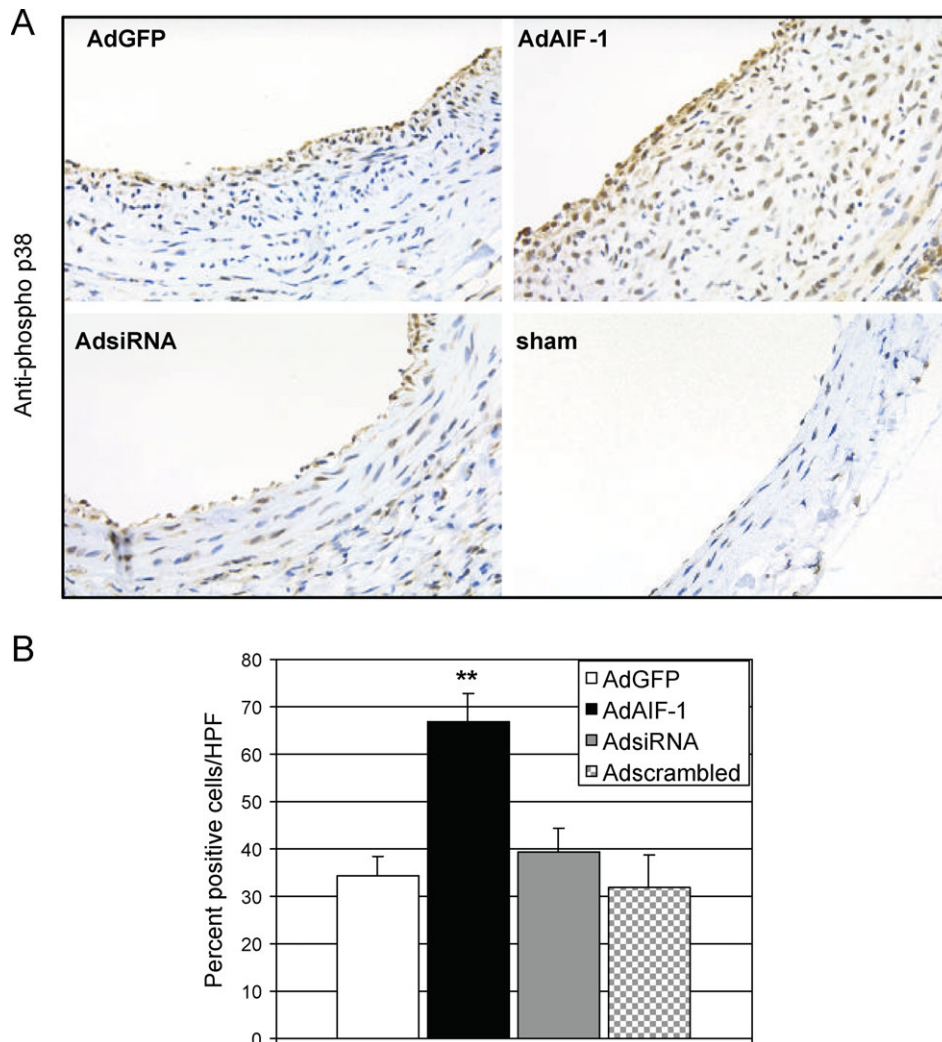
activation of cytoplasmic signal transduction proteins. Because of its molecular signature, we hypothesized that AIF-1 expression might regulate signal transduction pathways. Rat VSMCs were transduced with 40 MOI of AdGFP, AdAIF-1, or AdsiRNA and incubated in 0.25% FBS for 48 h. They were then stimulated with 10% FBS, and the phosphorylation state of p38 MAPK, an important mediator of proliferative and inflammatory signals, was evaluated by western analysis with phospho-specific p38 antibody. As expected, serum-stimulation increased p38 phosphorylation above baseline, unstimulated levels in both AdGFP- and AdAIF-1-transduced cells ( $P < 0.05$  for all time points). Overexpression of AIF-1 resulted in a significant increase in activation above AdGFP controls at serum-starved conditions ( $P < 0.05$ ,  $n = 4$ ). AIF-1 overexpression also resulted in sustained p38 phosphorylation and was significantly increased at 30 min post-stimulation vs. AdGFP controls ( $P < 0.01$ ,  $n = 4$ ) (Figure 4). In contrast, reduction of AIF-1 levels by AdsiRNA significantly reduced p38 activation back to baseline, unstimulated levels at 30 min post-stimulation, compared with AdGFP controls ( $P < 0.01$ ). No difference in p38 phosphorylation was noted in Adscrambled siRNA compared with AdGFP for any of the times (data not shown). Together, this suggests that in cultured VSMCs, AIF-1 overexpression is associated with increased and more sustained p38 phosphorylation, while the absence of AIF-1 results in a more transient activation of p38.

To translate these differences into the observed *in vivo* effects on intimal hyperplasia, immunohistochemistry of rat carotid arteries transduced with AdAIF-1, AdGFP, or

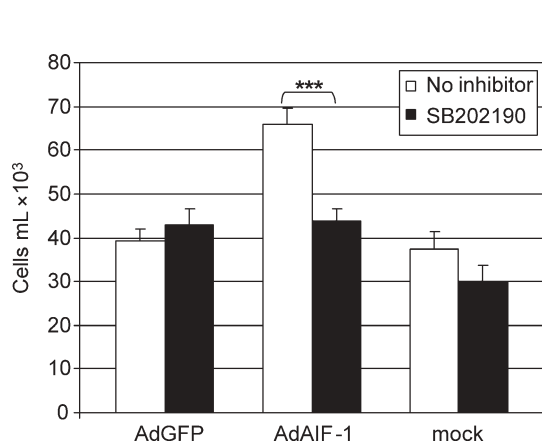
AdsiRNA was performed using antisera specific for phosphorylated p38 kinase. Percentage of positive cells was counted, rather than total number, to normalize for differences in size and cellularity neointima between groups. Figure 5 shows that compared with AdGFP controls, an increased percentage of neointimal cells stain positive for phosphorylated p38 in rat carotid arteries transduced with AdAIF-1 ( $34.4 \pm 4.1$  vs.  $67.0 \pm 5.8$  for AdGFP and AdAIF-1,  $P < 0.01$ ). Although the neointima size is much smaller, there is no significant difference in the percentage of cells which stain positive for phospho-p38 between AdGFP and AdsiRNA, or Adscrambled and AdGFP-transduced ( $39.5 \pm 5.0$ ,  $31.6 \pm 6.1$ , vs.  $34.4 \pm 4.1$  for AdsiRNA, Adscrambled, and AdGFP) vessels.

### 3.5 Allograft inflammatory factor-1 enhancement of proliferation, but not migration, is p38-dependent

The previous experiments in cultured VSMCs strongly suggested a relationship between AIF-1 expression and p38 activation. To determine whether AIF-1 proliferative effects are dependent on p38 activity, primary rat VSMCs were transduced with 40 MOI of AdGFP, AdAIF-1, or no adenovirus, and  $10 \mu\text{M}$  of the p38-specific inhibitor, SB202190, was added. After 5 days, VSMCs were trypsinized and counted. Five days were chosen to avoid potential effects of toxicity of the SB202190 with long-term exposure. Because AdsiRNA did not enhance proliferation, it was not included in this experiment. Figure 6 shows that neither



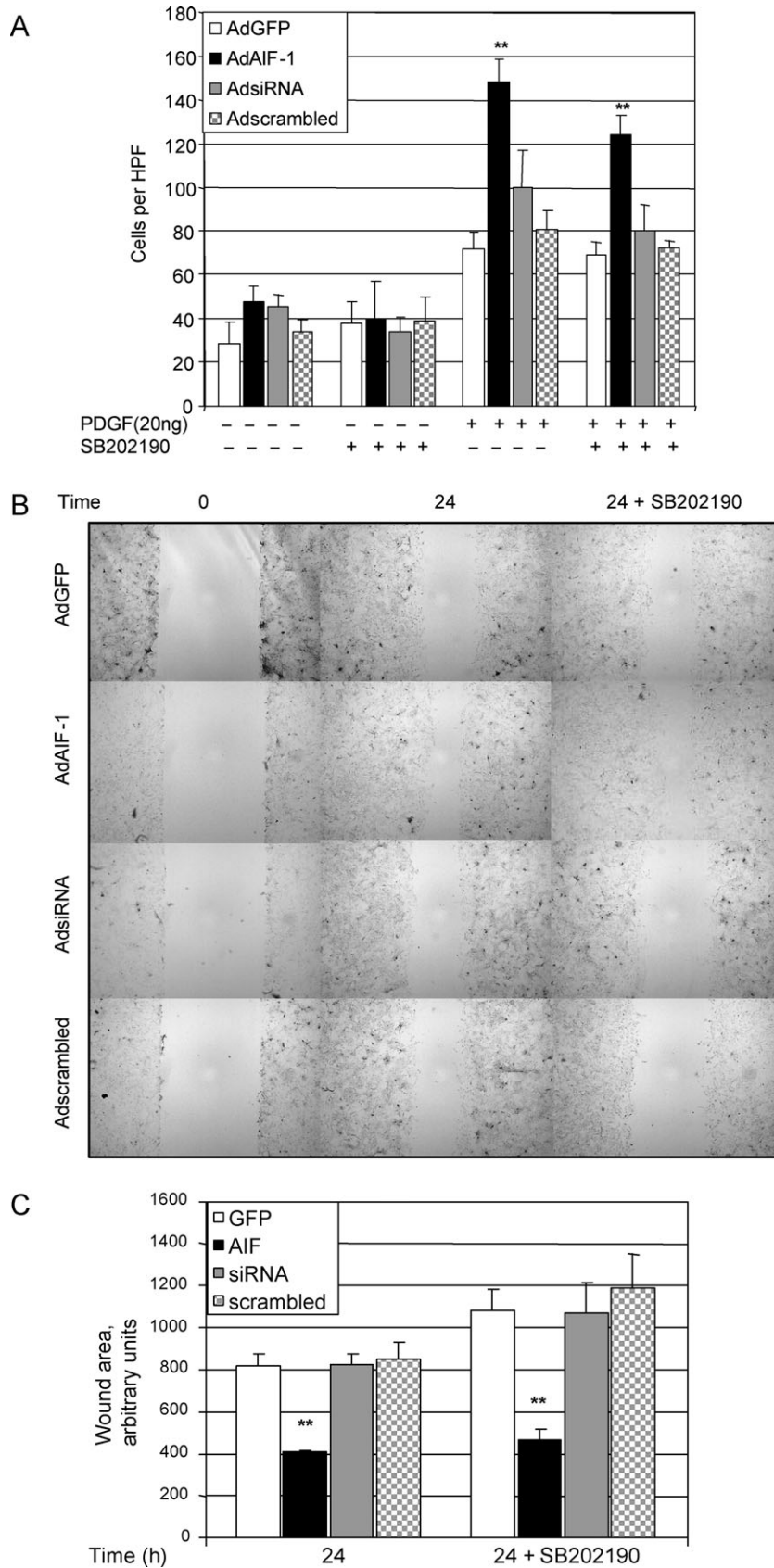
**Figure 5** Modulation of AIF-1 expression in injured arteries alters activation of p38 protein kinase. (A) Adenoviral delivery to balloon angioplasty-injured rat carotid arteries is identical to that described in *Figure 1*, immunostained with phospho-specific p38 protein kinase antibody,  $\times 400$  magnification. (B) Quantification and statistical analysis of phospho-p38 positive cells in rat carotid arteries were counted as percent positive cells from four high-powered fields. Asterisk indicates significant difference from AdGFP control ( $P < 0.01$ ). Errors are reported as SD.



**Figure 6** Inhibition of p38 MAPK abrogates allograft inflammatory factor-1 (AIF-1)-mediated proliferation. Primary rat VSMCs were transduced with 40 MOI of AdGFP, AdAIF-1, or no adenovirus. The next day,  $10 \mu\text{M}$  of the p38-specific inhibitor, SB202190, was added. After 5 days, VSMCs were counted. Shown are means from three independent transductions with similar results. Asterisk indicates significant difference from AdGFP control ( $P < 0.0001$ ).

AdGFP nor no adenovirus controls (mock) were inhibited by addition of p38 kinase inhibitor ( $39.8 \pm 2.6$ , and  $37.5 \pm 3.8$ , vs.  $42.7 \pm 3.7$  and  $30.1 \pm 4.7$ , for AdGFP and no adenovirus, without and with SB202190, respectively). In contrast, AIF-1-mediated increase in proliferation was almost completely abolished by inhibition of p38 ( $65.9 \pm 3.7$  vs.  $43.9 \pm 2.8$ , without and with SB202190, respectively,  $P < 0.001$ ). This suggests that the proliferative activities mediated by AIF-1 are dependent upon p38 MAPK activity.

We next determined whether migration-enhancing activity of AIF-1 utilized p38 MAPK activity. We performed two complementary experiments. First, we repeated Boyden chamber migration identical as we described for *Figure 3*, except some samples included the p38-specific inhibitor, SB202190. Inhibition of p38 has been shown to reduce PDGF-induced VSMC migration in several studies,<sup>21-23</sup> and all control and siRNA samples were significantly inhibited in the presence of this inhibitor ( $82 \pm 5.8$  vs.  $57.3 \pm 4.4$ ,  $100 \pm 5.4$  vs.  $64.3 \pm 11.6$ , and  $80.7 \pm 5.5$  vs.  $56.3 \pm 8.0$ , for AdGFP, AdsiRNA, and Adscrambled, PDGF vs. PDGF + SB202190, respectively,  $P < 0.05$  for all) (*Figure 7A*). In contrast to proliferation,



**Figure 7** Inhibition of p38 MAPK does not abrogate allograft inflammatory factor-1 (AIF-1)-mediated migration. Primary rat VSMCs were transduced with 40 MOI of AdGFP, AdAIF-1, or no adenovirus. (A) Boyden chamber migration as in Figure 3, with or without 10  $\mu$ M of the p38-specific inhibitor, SB202190. Values are means from three experiments performed in triplicate from three independently transduced groups of VSMCs. Asterisk indicates significant difference from AdGFP control ( $P < 0.01$ ). (B) Scratch wounding in the presence or absence of 10  $\mu$ M of SB202190. Cells were stained with haematoxylin and are magnified  $\times 40$ . This is representative of three independent experiments. (C) Quantitative analysis of scratch wound area from three different groups of adenoviral-infected VSMCs.

AIF-1-enhanced migration is p38 independent, as SB202190 has no significant effect on AIF-1-mediated VSMC migration ( $148 \pm 7.2$  vs.  $124 \pm 11.0$  cells/HPF for PDGF and PDGF + SB202190, respectively) (Figure 7A). AIF-1-expressing samples were significantly higher than controls ( $P < 0.001$  for all). We also performed a scratch wound assay in which directional migration of VSMC monolayers in the presence and absence of SB202190 could be assessed. Equal numbers of confluent VSMCs infected with either AdGFP, AdAIF-1, AdsiRNA, or Adscrambled were incubated in growth media and were scraped to create a 3 mm wide wound track devoid of cells. The data presented in Figure 7B demonstrate that AIF-1 cells migrate into the wound at a more rapid rate than do control or AdsiRNA-infected cells. Similar to previous experiments, AdsiRNA-expressing cells show no decrease in directional migration compared with controls. Also similar to Boyden chamber migration, the inclusion of SB202190 does not decrease the AIF-1-enhanced directional migration of VSMCs. This suggests that the inhibition of p38 MAPK does not have deleterious effects on AIF-1 enhancement of VSMC migration.

#### 4. Discussion

Work from several investigators demonstrate AIF-1 expression in diverse inflammatory situations suggest that AIF-1 expression is a fundamental component of the inflammatory response. We have previously shown that AIF-1 expression is undetectable in uninjured arteries but strongly induced in arterial VSMCs by injury, and AIF-1 is detectable at low levels in cultured VSMCs, but strongly upregulated by inflammatory cytokines.<sup>11</sup> Although forced AIF-1 expression increases migration and proliferation of cultured human VSMCs, and chronic AIF-1 overexpression enhanced intimal hyperplasia in transgenic mice, the effects of AIF-1 abrogation on development of intimal hyperplasia has not been reported.<sup>14,15</sup> As such, one goal of this study was to determine whether inhibition of AIF-1 expression could reduce the severity of neointimal formation. Using adenoviral delivery of AIF-1 cDNA and AIF-1 siRNA, respectively, to balloon angioplasty-injured rat carotid arteries, we observed that overexpression of AIF-1 significantly increased neointimal formation, compared with GFP and vehicle only controls, whereas reduction of AIF-1 significantly decreased neointimal formation.

Neointimal formation in response to injury in the rat carotid model is primarily a result of VSMC migration and proliferation in the lumen.<sup>24</sup> The effects of AIF-1 knockdown on VSMC migration and proliferation of those parameters have not been investigated. In this study, overexpression significantly increased both proliferation and migration. Consistent with that, reduction of AIF-1 expression by siRNA significantly reduced VSMC proliferation. Conversely, siRNA knockdown of AIF-1 expression did not significantly inhibit migration. While this suggests that *in vivo*, AIF-1 enhancement of neointimal formation is primarily due to proliferative effects, it could also imply that AIF-1 expression is necessary and sufficient to enhance proliferative, but not migratory pathways. An alternative, though not exclusive, explanation is in the marked differences between *in vivo* response to injury and *in vitro* migration assay. For example, although low levels of AIF-1 are detectable in serum-starved cultured VSMCs, the 3 h of PDGF stimulation

in the migration assay is not sufficient time to induce AIF-1 expression, and thus the AdsiRNA knockdown would have very limited effects. Conversely, when VSMCs are cultured for several days as in the proliferation assay, AIF-1 expression is induced, and thus the impact of AdsiRNA is much more pronounced. AIF-1 may not play a role in normal VSMC function because there is little AIF-1 expression in uninjured arteries, or unstimulated cultured VSMCs, which also may explain why AIF-1 knockdown effects are not dramatic in cultured cells. When AIF-1 is over-expressed as it is in response to injury, we do see similarities between *in vivo* and *in vitro* assays. Local inflammation plays an important role in the development of the neointima following vascular injury. Immunostaining with CD45 antibody, which recognizes leucocyte common antigen, did not demonstrate any significant differences in AdAIF-1, AdGFP, AdsiRNA, and Adscrambled arteries (data not shown). Their numbers were so few as to preclude power enough for valid statistical analysis. This may not be surprising, considering that leucocyte infiltration in the angioplasty-injured rat carotid is very limited in comparison with rabbits and swine, and this model is considered to be highly proliferative, rather than inflammatory in nature.<sup>25,26</sup> Thus, we cannot completely rule out inflammatory effects of AIF-1 expression on development of intimal hyperplasia.

Arterial injury modulates the activity of several MAPK family members, including extracellular signal regulated kinase-1/2, (ERK1/2), p38, JNK, and stress-activated protein kinase.<sup>2,3,27</sup> Further, compounds that reduced or inhibited the activity of these kinases also attenuate VSMC activation and neointimal injury after arterial injury.<sup>6,28</sup> AIF-1 has signatures of a cytoplasmic signaling protein, including several PDZ interaction domains, which are important in mediating interactions of multi-protein complexes.<sup>13</sup> Chronic overexpression of AIF-1 in transgenic murine VSMC activated p38.<sup>17</sup> In this study in cultured rat VSMCs, a more direct relationship between AIF-1 expression levels and p38 activation was shown. Not only did increased AIF-1 result in increased levels of p38 phosphorylation in unstimulated VSMCs, but also a more sustained phosphorylation. In contrast, reduction of AIF-1 expression resulted in a more transient p38 activation in that phosphorylation returned to baseline levels by 30 min post-stimulation.

Allograft inflammatory factor-1 effects on p38 activation are noteworthy in that p38 MAPK is an inflammation-responsive member of the MAPK superfamily and mediates activation of VSMCs in response to injury and inflammation.<sup>21,29,30</sup> Use of multiple, p38-specific pharmacological inhibitors has shown that sustained activation of p38 is an important contributor to the vascular response to injury.<sup>7,31</sup> In a previous report in murine macrophages, we found that AIF-1 knockdown decreased activation of ERK1/2 MAPK by stimulation with oxidized LDL and inflammatory stimuli.<sup>19</sup> In contrast to murine monocytes, p44/42 MAPK activation in rat VSMCs was not reduced by knockdown of AIF-1 expression (data not shown).<sup>19</sup>

p38 MAPK remains elevated up to 14 weeks post-balloon angioplasty.<sup>7,32</sup> Use of phospho-specific antibody allowed us to complement data from cultured VSMCs with immunohistochemical analysis of angioplasty-injured rat carotid arteries in which AIF-1 expression had been altered with adenovirus. AIF-1 overexpression increased p38



phosphorylation in injured arteries, which is consistent with the sustained p38 phosphorylation observed in cultured VSMCs. In contrast to *ex vivo* studies, AIF-1 knockdown did not reduce p38 phosphorylation below control levels. The most likely reason for this is the obvious differences in the *in vivo* response to injury and the more controlled environment of cultured VSMCs. The *in vivo* situation, in which VSMCs exist in a milieu of inflammatory and proliferative stimuli, is much more complex than cultured VSMCs which had been synchronized by serum starvation and a defined stimulation by foetal calf serum. It has been speculated that the induction of growth factors are more long lasting and remain elevated in the neointima up to 14 days post-injury.<sup>5</sup> The multiple stimuli originating from inflammatory cells and their multiple effects on VSMCs could contribute to p38 activation in AIF-1-independent pathways. An additional point to consider is the limited efficacy of adenoviral delivered siRNA 14 days post-injury. The mechanism of AIF-1 enhancement of p38 activity remains unclear. Although AIF-1 does not directly interact with p38 (data not shown), AIF-1 overexpression does activate Rac1.<sup>15</sup> In some SMCs, Rac1 is an upstream mediator of p38.<sup>29,33</sup> Thus, AIF-1 effects on proximal mediators of p38 activation may account for the relationship between AIF-1 expression and p38 activity.

It has been shown that selective inhibition of p38 does not inhibit PDGF-driven VSMC proliferation.<sup>7</sup> Similarly, in the present study, SB202190 does not inhibit AdGFP or untreated control VSMCs, but almost completely abolishes the proliferative effects imparted by AIF-1 are dependent upon p38 activity. Our findings that the inhibition of p38 has no inhibitory effect on AIF-1 migratory activity were unexpected. This may have to do with the fact that the Boyden chamber assay is rather rapid. However, the scratch wound assay, performed in media containing 10% FBS and carried out for 24 h corroborated the Boyden chamber results. This strongly suggests that AIF-1 effects on migration are p38 independent. As AIF-1 expression is induced in response to inflammatory stimuli, and p38 is an important integrator of inflammatory signals, this implies that AIF-1 is a functional part of the inflammation-mediated p38 MAPK signaling pathway leading at least to proliferation.<sup>11</sup> These data suggest that AIF-1 activity may be proximal to, or at least at the level of p38 MAPK for two reasons; (i) overexpression of AIF-1 activates p38, suggesting that AIF-1 is upstream of p38 and (ii) blocking p38 greatly reduces AIF-1 proliferation-enhancing effects. AIF-1 does contain an MAPK interaction motif [(R/K)xxx#x# where # is a hydrophobic residue], but we have not been able to show a direct interaction between AIF-1 and p38. Consequently, at this time, the mechanism for these effects remains unclear.

There are at least five novel points to this study: (i) AIF-1 overexpression enhances and inhibition of AIF-1 expression reduces intimal hyperplasia in response to injury, (ii) AIF-1 overexpression enhances and under expression reduces VSMC proliferation, (iii) AIF-1 overexpression enhances and sustains p38 phosphorylation *in vivo* and in cultured VSMCs, but p38 expression is more transient when AIF-1 expression is reduced, (iv) AIF-1 overexpression enhances VSMC migration, but the inhibition of AIF-1 does not reduce migration, and (v) AIF-1 enhancement of VSMC

proliferation is p38 dependent, but AIF-1 enhancement of migration is p38 independent. Based on its molecular structure and expression profile, one explanation for the induction of AIF-1 expression in vascular injury is to function as a scaffold to bring one or more signal transduction kinases into reactive proximity with its specific substrate in response to vascular inflammation. These data, together with previously published studies, support our central hypothesis that AIF-1 is an inflammation-responsive signaling protein that plays an important role in the regulation of VSMC activation and development of vasculopathies associated with vascular injury.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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