Ablation of MEK Kinase 1 Suppresses Intimal Hyperplasia by Impairing Smooth Muscle Cell Migration and Urokinase Plasminogen Activator Expression in a Mouse Blood-Flow Cessation Model

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Background—Migration, proliferation, and matrix-degrading protease expression of smooth muscle cells (SMCs) are major features of intimal hyperplasia after vascular injury. Although MEK kinase 1 (MEKK1) has been shown to regulate cell migration and urokinase plasminogen activator (uPA) expression, the precise role of MEKK1 in this process remains unknown.

Methods and Results—We triggered a vascular remodeling model by complete ligation of the right common carotid artery in wild-type (WT) and MEKK1-null (MEKK1−/−) mice. The intimal areas 28 days after ligation were significantly decreased in the ligated MEKK1−/− arteries compared with WT arteries (28±8 versus 65±17 μm², P<0.05). There were no differences in the ratios of proliferating cell nuclear antigen (PCNA)-positive cells to total cells within the arterial wall between WT and MEKK1−/− arteries. Proliferation capacity also did not differ between WT and MEKK1−/− cultured aortic smooth muscle cells (AoSMCs). In contrast, the number of intimal PCNA-positive cells 7 days after ligation was significantly smaller in MEKK1−/− arteries. Three different migration assays revealed that migration and invasion of MEKK1−/− AoSMCs were markedly impaired. Addition of full-length MEKK1 restored the migration capacity of MEKK1−/− AoSMCs. The number of MEKK1−/− AoSMCs showing lamellipodia formation by epithelial growth factor was significantly smaller compared with those of WT SMCs. Furthermore, uPA expression after ligation was markedly decreased in MEKK1−/− arteries.

Conclusions—MEKK1 is implicated in vascular remodeling after blood-flow cessation by regulating the migration and uPA expression of SMCs. MEKK1 is a potential target for drug development to prevent vascular remodeling. (Circulation. 2005;111:1672-1678.)

Key Words: remodeling ■ muscle, smooth ■ vasculature ■ restenosis

Blood vessels respond to damaging stimuli by activating a remodeling mechanism that leads to intimal hyperplasia.1,2 Accumulating evidence has shown that the underlying causes of intimal hyperplasia are the invasion and proliferation of vascular smooth muscle cells (SMCs), both of which processes are triggered and controlled by numerous growth factors and mitogens.3 Cell invasion involves migration by cytoskeletal reorganization and activation of a cascade of proteases that degrade various extracellular matrix (ECM) components.4 Urokinase-type plasminogen activator (uPA) is responsible for degradation of the ECM.4 Recent studies in uPA-deficient mice have demonstrated that the number of neointimal SMCs after injury is markedly reduced compared with those in wild-type (WT) mice, suggesting that uPA plays a critical role in cell invasion during vascular remodeling.5

MEK kinase 1 (MEKK1) is a 196-kDa, mitogen-activated protein kinase kinase (MAP3K) that acts as an upstream regulator of several MAPK pathways.6-8 MEKK1 has been implicated in diverse and cell type–specific biological responses, including cardiac hypertrophy,6 cell survival,7

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1672
and apoptosis. Recent studies on MEKK1-null (MEKK1<sup>−/−</sup>) mice have uncovered a unique function for this protein kinase in cell migration. MEKK1<sup>−/−</sup> mice were found to exhibit impairment of embryonic eyelid closure, a process involving epithelial cell migration. MEKK1 is also involved in growth factor–induced embryonic stem cell migration and contributes to fibroblast and epithelial cell migration in vitro. Furthermore, endogenous and overexpressed MEKK1 was reported to colocalize with the α-actinin cytoskeleton along actin stress fibers in focal adhesions. Conversely, cytoskeletal reorganization can also lead to MEKK1 activation. This close relationship between MEKK1 and the cytoskeleton implies that MEKK1 might play an important role in SMC migration and uPA production, both of which will affect SMC invasion during vascular remodeling. Herein, using a blood-flow cessation model in mice with targeted ablation of the MEKK1 gene, we tested our hypothesis that MEKK1 plays a pivotal pathophysiological role in arterial remodeling by regulating SMC migration and uPA expression.

**Results**

**Primary Culture of AoSMCs and Transfection**

Primary culture of aortic SMCs (AoSMCs) was performed and characterized as described previously. Cells were identified by positron immunostaining for α-smooth muscle actin (American Research Products). AoSMCs were transiently transfected with lipofectamine and either a full-length form of MEKK1 vector (MEKK1FL) or an empty vector (pcDNA3.1). After 48 hours, AoSMCs were transiently transfected with lipofectamine and either a full-length form of MEKK1 vector (MEKK1FL) or an empty vector (pcDNA3.1). After 48 hours, AoSMCs were transiently transfected with lipofectamine and either a full-length form of MEKK1 vector (MEKK1FL) or an empty vector (pcDNA3.1).

**AoSMC Proliferation Assay**

AoSMCs (<5×10<sup>4</sup>) were cultured for 24 hours in 96-well plates with or without epithelial growth factor (EGF, 20 ng/mL), FGF-2 (20 ng/mL, R&D Systems, Inc), or platelet-derived growth factor-BB (PDGF-BB, 25 ng/mL, Sigma). Then, [3H]thymidine incorporation and cell number were measured, as previously reported.

**Quantification of Scrape Wound–Induced Migration Assay**

Dense monolayers of overconfluent AoSMCs grown on Laboratory-Tek chamber slides were scraped with a sterile scraper as described previously. The wound was created, cells were incubated for 24 hours with or without FGF-2 (20 ng/mL) and visualized with rhodamine-phalloidin (R-451, Molecular Probes). The number of invading cells was manually counted per high-power field for each condition (10 fields for each filter). The percentage of invasion was calculated as (invading cells in Matrigel inserts/migrated cells of control inserts) × 100.

**Immunofluorescence Confocal Microscopy**

Immunofluorescence staining with rhodamine-phalloidin and a monoclonal antibody for uPA with rhodamine-phalloidin and a monoclonal antibody for uPA, as described previously. Staining was examined with a Nikon Eclipse Te2000-U confocal scanning electron microscope.

**Protein Extraction and Western Blotting Analysis**

Protein extraction and immunoblotting were performed as described previously. Protein phosphorylation levels were normalized to the matching densitometric values of nonphosphorylated proteins.

**Statistical Analysis**

All data were expressed as mean±SEM. Significant differences were analyzed by an unpaired Student t test, Fisher exact test, or ANOVA followed by the Bonferroni post hoc test. A value of P<0.05 was considered statistically significant.

**Results**

**MEKK1 Ablation Inhibits Intimal Hyperplasia**

There was no intimal thickening in unligated arteries of either WT or MEKK1<sup>−/−</sup> mice. Significant neointimal growth was observed 28 days after ligation in WT mice, whereas it was much less in MEKK1<sup>−/−</sup> mice (Figure 1A). Morphometric analysis of ligated arteries on day 28 revealed that the average intimal area was significantly smaller in MEKK1<sup>−/−</sup> mice than in WT mice, whereas no difference in medial area was observed between WT and MEKK1<sup>−/−</sup> mice (Figure 1B). Consequently, I-M ratios and stenotic ratios were significantly decreased in MEKK1<sup>−/−</sup> mice compared with those in WT mice (Figure 1B).

**MEKK1 Ablation Prevented Increases in the Numbers of Intimal PCNA-Positive Cells**

In both WT and MEKK1<sup>−/−</sup> mice, the ratios of PCNA-positive cells to total cells within the arterial wall, intima, and media were significantly increased 3 days after ligation.
compared with those 1 day after ligation, but there were no significant differences between WT and MEKK1<sup>−/−</sup> mice. It is noteworthy that 7 days after ligation, the number of PCNA-positive cells in the intima was significantly less in MEKK1<sup>−/−</sup> arteries than in WT arteries, although there was no difference within other areas (Figure 2A and 2B). These findings suggest that migration of PCNA-positive cells from the media to the intima was impaired, whereas proliferation was not impaired in MEKK1<sup>−/−</sup> mice. Consistent with in vivo data, no difference was observed in [3H]thymidine incorporation and cell number between WT and MEKK1<sup>−/−</sup> AoSMCs after stimulation with PDGF-BB in vitro (Figure 2C). Treatment with EGF or FGF-2 instead of PDGF-BB yielded identical results (data not shown).

**Effects of Ablation of MEKK1 on MAPK Activities of AoSMCs**

Ablation of MEKK1 does not affect the total protein expression of extracellular signal–regulated kinase (ERK), c-Jun NH<sub>2</sub>-terminal kinase (JNK), or p38. We examined the effects of ablation of MEKK1 on MAPK activities of AoSMCs. FGF-2–induced JNK and ERK but not p38 activation in AoSMCs from MEKK1<sup>−/−</sup> mice was less than that from WT mice (Figure 3A). We confirmed that addition of MEKK1 restored MEKK1 protein levels in MEKK1<sup>−/−</sup> AoSMCs (Figure 3B). Addition of MEKK1 restored JNK and ERK activation in response to FGF-2 in MEKK1<sup>−/−</sup> AoSMCs (Figure 3C and 3D). Treatment with EGF or PDGF-BB instead of FGF-2 yielded identical results (data not shown).

**MEKK1 Ablation Inhibited AoSMC Migration and Invasion**

In the scrape wound–induced migration assays, the average number of and distance that MEKK1<sup>−/−</sup> AoSMCs migrated from the wound edge (white dotted line) in the absence of any stimulus was similar to those in WT mice; however, the FGF-2–induced increase in cell number and distance was significantly suppressed in MEKK1<sup>−/−</sup> AoSMCs compared with WT cells. Addition of MEKK1 restored the number and distance of MEKK1<sup>−/−</sup> AoSMCs to normal levels (Figure 4A and 4B).

In the aortic explant assays, the number of AoSMCs migrating from MEKK1<sup>−/−</sup> explants at 10 days was considerably lower than those from WT explants (Figure 4C). Moreover, the number of MEKK1<sup>−/−</sup> aortic explants that showed migrating cells was significantly smaller than in WT explants (27.2±2.2% versus 60.0±2.5%, P<0.05).

In the transwell Matrigel-coated chamber invasion assays, the number of invading MEKK1<sup>−/−</sup> AoSMCs in response to FGF-2 was suppressed markedly compared with WT cells. Addition of MEKK1 restored the number of MEKK1<sup>−/−</sup> AoSMCs to normal levels (Figure 4D). Treatment with EGF or PDGF-BB instead of FGF-2 yielded identical results (data not shown).

**MEKK1 Ablation Impaired Lamellipodia Formation**

There were no morphological differences between WT and MEKK1<sup>−/−</sup> AoSMCs under basal conditions; however, typi-
cal lamellipodia formation was induced by treatment with EGF in WT AoSMCs but was seldom induced in MEKK1−/− AoSMCs (Figure 5A). The percentage of MEKK1−/− AoSMCs showing lamellipodia was significantly lower than in WT AoSMCs (P < 0.05). Addition of MEKK1 restored the lamellipodia-forming capacity of MEKK1−/− AoSMCs (Figure 5B). Treatment with FGF-2 or PDGF-BB instead of EGF yielded identical results (data not shown).

Figure 3. Effects of ablation of MEKK1 on MAPK activities in AoSMCs. A, Time course of JNK, ERK, and p38 activities followed by FGF-2 stimulation. B, Addition of MEKK1 experiments in MEKK1−/− AoSMCs transfected with empty vector (pcDNA3.1) or full-length forms of MEKK1 vector (MEKK1FL) followed by FGF-2 stimulation. C, Western blot analysis of MAPKs in MEKK1−/− AoSMCs transfected with empty vector (pcDNA3.1) or full-length forms of MEKK1 vector (MEKK1FL) followed by FGF-2 stimulation. D, Cumulative data for MAPK in Figure 3C. *P<0.05 vs WT AoSMCs.

MEKK1 Ablation Decreased uPA Expression

uPA expression began to increase on day 1 and reached a peak on day 3, after which it decreased gradually by 7 days after ligation in WT mice. In contrast, there was only weak positive staining for uPA up to 7 days in MEKK1−/− mice (Figure 5A). The percentage of MEKK1−/− AoSMCs showing lamellipodia was significantly lower than in WT AoSMCs (P < 0.05). Addition of MEKK1 restored the lamellipodia-forming capacity of MEKK1−/− AoSMCs (Figure 5B). Treatment with FGF-2 or PDGF-BB instead of EGF yielded identical results (data not shown).

Figure 4. Migration and invasion of AoSMCs. A, Scrape wound-induced migration assay. Immunofluorescence microscopy of migrating cells from wound edge when AoSMCs were cultured for 24 hours with or without FGF-2. F-actin was stained with rhodamine-phalloidin (red), and nuclei were counterstained with DAPI (blue; not visible). B, Quantification of means of number and distance of migrating cells. *P<0.05 vs WT AoSMCs. C, Migration of AoSMCs from aortic explants on day 10. D, Quantification of percent invasion of AoSMCs by transwell Matrigel-coated chamber invasion assay. *P<0.05 vs WT AoSMCs.

Discussion

Several lines of evidence suggest that MEKK1 is implicated in diverse biological responses.6–8 Recently, the unique role of MEKK1 in regulating the migration of several cell types has aroused widespread attention9,12,27 and inspired us to study its involvement in cardiovascular diseases. Up to now, there has been no previous report on the role of MEKK1 in the development of vascular remodeling, during which invasion and proliferation of SMCs play key roles. In the present study, we investigated the mechanism whereby MEKK1 regulates vascular remodeling in a well-established, blood-flow cessation model in MEKK1−/− mice. We found that MEKK1 is essential for intimal hyperplasia after cessation of blood flow.

In this study, we clearly demonstrated that intimal areas, I-M ratios, and stenotic ratios of the ligated arteries were significantly lower in MEKK1−/− mice relative to WT mice, indicating that MEKK1 is implicated in intimal hyperplasia. Although an angioplasty/balloon injury model would have yielded greater applicability to the clinical situation, we used...
the ligation model because of its excellent reproducibility. To clarify the mechanism(s) of MEKK1 involvement, we first examined cell proliferation within the arterial wall after ligation and found no statistically significant difference in the number of PCNA-positive cells between WT and MEKK1⁻/⁻ mice. Results from this ligation model are consistent with the fact that MEKK1⁻/⁻ mice have no overt defects in growth and fertility.⁹ There was also no difference in the proliferation of WT and MEKK1⁻/⁻ AoSMCs evaluated by [³H]thymidine incorporation and cell number. These findings suggest that SMC proliferation might not contribute to the reduced intimal hyperplasia in MEKK1⁻/⁻ mice. Then we investigated SMC migration and uPA expression, both of which are important factors for intimal hyperplasia.

We demonstrated that there were significantly fewer intimal PCNA-positive cells 7 days after ligation in MEKK1⁻/⁻ mice than in WT mice. Because PCNA-positive cells are believed to migrate from the media to the intima,² this finding suggests that SMC migration is impaired in MEKK1⁻/⁻ mice. To directly assess the effects of MEKK1 ablation on SMC migration, we used several migration and invasion assays in vitro. We observed comparable migration or invasion of SMCs under control condition between WT and MEKK1⁻/⁻ AoSMCs; however, significant impairment of migration or invasion was observed after stimulation with FGF-2 in MEKK1⁻/⁻ AoSMCs, which was restored by addition of full-length MEKK1. These findings indicate that ablation of MEKK1 impairs invasion and migration, both of which may contribute to reduced intimal hyperplasia wherein growth factors may play a vital role.

Lamellipodia formation is essential for cell migration.¹³ Exogenous stimuli, such as PDGF or FGF-2, induce lamellipodia formation that can help to complete the first step of the motility cycle.¹⁴,²⁸ Therefore, we examined whether MEKK1 is involved in lamellipodia formation in AoSMCs. Our results showed that the percentage of lamellipodia-positive cells was significantly smaller in MEKK1⁻/⁻ AoSMCs compared with WT AoSMCs in the presence of EGF. This finding suggests that the impairment of migration in MEKK1⁻/⁻ AoSMCs may be due to inhibited formation of lamellipodia. Indeed, addition of MEKK1 to MEKK1⁻/⁻ AoSMCs recovered their capacity to form lamellipodia and migrate. On the other hand, it has been reported that loss of MEKK1 disrupts focal adhesion composition, with decreased vinculin content and focal adhesion kinase (FAK) cleavage.²⁹ Because disruption of focal adhesion composition will affect cell migration, further investigation will be needed to clarify its role in MEKK1⁻/⁻ mice.

There is evidence that uPA is induced after arterial injury.⁵,²⁸,³⁰,³¹ uPA enhances vascular remodeling by transforming plasminogen into plasmin, which can activate metalloproteinases and in turn degrade ECM proteins.⁵,³⁰,³¹ We found that uPA expression began to increase at 1 day and reached a peak 3 days after ligation in WT mice, whereas uPA staining appeared to be significantly lower in MEKK1⁻/⁻ arteries at corresponding times. In vitro, PDGF-BB– and FGF-2–induced uPA expression as detected by immunofluorescence staining and Western blotting was also significantly decreased in MEKK1⁻/⁻ AoSMCs. Thus, in addition to impaired lamellipodia formation, inhibited uPA expression by MEKK1 ablation may also contribute to impairment of SMC invasion. Addition of full-length MEKK1 restored uPA expression in MEKK1⁻/⁻ AoSMCs.
Although it has been reported that MEK1 is required for FGF-2–induced signals to control uPA expression in fibroblasts,15 further investigations will be needed to elucidate the mechanism by which MEK1 regulates uPA expression in arteries after ligation.

Recent studies have demonstrated that JNK and ERK transduction pathways may regulate cell migration32,33 and uPA expression.34,35 In the present study, we demonstrated that JNK and ERK activation after growth factor stimulation was blunted in MEK1−/− AoSMCs. Thus, it is possible that ablation of MEK1 may inhibit cell migration and uPA expression by interfering with the downstream signaling pathways JNK and/or ERK. MEK1 also has been reported to be associated with cytoskeletal reorganization11,12 and to be necessary for uPA upregulation,15 suggesting another possibility that ablation of MEK1 directly inhibits lamellipodia formation and uPA expression. The stimulus for remodeling after ligation is also influenced by the resultant vascular ischemia. Because MEK1 is activated by hypoxic stimuli as well as growth factors,36 we must consider the possibility that the resultant hypoxic stimuli are also important during vascular remodeling in the ligation model.

Izumi et al23 demonstrated that activation of apoptosis signal–regulating kinase 1 (ASK1), another member of the MAP3K family, also plays a key role during intimal hyperplasia in the carotid artery balloon injury model. Unlike MEK1, ablation of ASK1 blunted both JNK and p38 but not ERK activation in AoSMCs after serum stimulation. In addition, ablation of ASK1 caused impairment of both SMC migration and proliferation. Thus, although the methods or models used to evaluate functions of MEK1 and ASK1 were not the same, both MEK1 and ASK1 may contribute to the development of intimal hyperplasia by different mechanisms.

In conclusion, we have demonstrated that MEK1 plays a critical role during intimal hyperplasia in a mouse carotid blood-flow cessation model. Intimal hyperplasia is greatly lessened, possibly due to a reduction of SMC invasion by an impairment of their migration and reduced uPA expression. We propose that MEK1 is a potential target for drug development to prevent vascular remodeling.

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14. DesMarais V, Ichetovkin I, Garrington TP, Schlesinger TK, Gibson S, Johnson GL. MEK kinase 1 (MEK1) regulates cell migration and proliferation. Thus, although the methods or models used to evaluate functions of MEK1 and ASK1 were not the same, both MEK1 and ASK1 may contribute to the development of intimal hyperplasia by different mechanisms.

In conclusion, we have demonstrated that MEK1 plays a critical role during intimal hyperplasia in a mouse carotid artery balloon injury model. Unlike MEK1, ablation of ASK1 blunted both JNK and p38 but not ERK activation in AoSMCs after serum stimulation. In addition, ablation of ASK1 caused impairment of both SMC migration and proliferation. Thus, although the methods or models used to evaluate functions of MEK1 and ASK1 were not the same, both MEK1 and ASK1 may contribute to the development of intimal hyperplasia by different mechanisms.

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