Activation of Apoptosis Signal-Regulating Kinase 1 in Injured Artery and Its Critical Role in Neointimal Hyperplasia

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Background—Apoptosis signal-regulating kinase 1 (ASK1), recently identified as one of the mitogen-activated protein kinase kinase kinases, is activated by various extracellular stimuli and involved in a variety of cellular functions. Therefore, we first examined the role of ASK1 in vascular remodeling.

Methods and Results—We used rat balloon injury model and cultured vascular smooth muscle cells (VSMCs). Arterial ASK1 activity was rapidly and dramatically increased after balloon injury. To specifically inhibit endogenous ASK1 activation, dominant-negative mutant of ASK1 (DN-ASK1) was transfected into rat carotid artery before balloon injury. Gene transfer of DN-ASK1 significantly prevented neointimal formation at 14 days after injury. Bromodeoxyuridine labeling index at 7 days after injury showed that DN-ASK1 remarkably suppressed VSMC proliferation in both the intima and the media. We also examined the role of ASK1 in cultured rat VSMCs. Infection with DN-ASK1 significantly attenuated serum-induced VSMC proliferation and migration. We also compared neointimal formation after cuff placement around the femoral artery between mice deficient in ASK1 (ASK1−/− mice) and wild-type (WT) mice. Neointimal formation at 28 days after cuff injury in ASK1−/− mice was significantly attenuated compared with WT mice. Furthermore, we compared the proliferation and migration of VSMCs isolated from ASK1−/− mice with WT mice. Both proliferation and migration of VSMCs from ASK1−/− mice were significantly attenuated compared with VSMCs from WT mice.

Conclusions—ASK1 activation plays the key role in vascular intimal hyperplasia. ASK1 may provide the basis for the development of new therapeutic strategy for vascular diseases. (Circulation. 2003;108:2812-2818.)

Key Words: balloon ■ muscle, smooth ■ remodeling ■ signal transduction

Vascular injury induces vascular smooth muscle cell (VSMC) proliferation with abundant subsequent production of extracellular matrix.1,2 Accumulating evidence indicates that intimal thickening by balloon injury is mediated by a complex interaction of a variety of growth-regulatory molecules, such as growth factors, vasoactive peptides, inflammatory cytokines, or chemokines,3–5 and these molecules are therefore regarded as the useful target for treatment of vascular thickening. The notion that targeting the critical common intracellular signals, activated by various extracellular stimuli, may be more useful than the inhibition of a single regulatory pathway has been proposed by recent experimental findings on the prevention of neointimal hyperplasia by in vivo transfer of antisense oligonucleotides of cdc2 kinase or proliferating cell nuclear antigen, retinoblastoma protein RB, cyclin kinase inhibitor protein p21, or various dominant-negative mutants of signaling molecules, as reviewed.6–8 Despite the critical and diverse role of intracellular protein kinase cascades in cellular responses by the above-mentioned neointima-associated molecules, the role of the protein kinases in vascular thickening remains to be fully understood.

Apoptosis signal-regulating kinase 1 (ASK1), identified as one of mitogen-activated protein kinase kinase kinases (MAPKKKs), is activated in response to proinflammatory and various stress signals and relays those signals to the downstream of mitogen-activated protein (MAP) kinase cascades.9–11 ASK1 has been initially identified as an apoptosis-inducing kinase10,12 and recently has also reported to be implicated in a variety of cellular functions, including cell survival,13 differentiation,14 and inflammatory response.15

In the present study, we investigated the role of ASK1 in vascular remodeling after injury by using adenoviral gene transfer technique and mice deficient in ASK1 (ASK1−/− mice).

Methods

Animals

All procedures were in accordance with institutional guidelines for animal research. Male Sprague-Dawley rats (Clea Japan, Tokyo, Osaka 545-8585, Japan. E-mail kims@med.osaka-cu.ac.jp © 2003 American Heart Association, Inc. Circulation is available at http://www.circulationaha.orgDOI: 10.1161/01.CIR.0000096486.01652.FC
Japan) weighing 300 to 350 g and male ASK1−/− mice17 and wild-type (WT) mice from the same genetic background (C57BL/6J) were used in the present study.

Construction of Recombinant Adenovirus
Adenovirus vectors expressing WT ASK1 (W-ASK1) and dominant-negative mutant of ASK1 (DN-ASK1) (Lys 709 to Met), tagged hemagglutinin (HA) in the site of NH2-terminus, have been described previously.10

In Vivo Gene Transfer and Balloon Injury
In vivo gene transfer to rat carotid artery was performed, as described in detail.16,17 DN-ASK1, W-ASK1, or empty-vector (Ad-E) as control (each 1×109 plaque-forming units) was infused into the left common carotid artery. At 2 days after gene transfer, the endothelial denudation of the common carotid artery was carried out, as previously described.17

Preparation of Arterial Protein Extracts and Western Blot Analysis
Our detailed method has been previously described.16,18 After electrophoretic transfer, the membranes were probed with antibodies to phospho-ASK1, phospho-p38 MAPK (p38), phospho-c-Jun NH2-terminal kinase (JNK), or phospho–extracellular signal-regulated kinase (ERK). All antibodies except for phospho-ASK1 were purchased from New England Biolabs, Inc.

Morphometric Analyses, Cell Proliferation, and Terminal Deoxynucleotidyl Transferase–Mediated dUTP–Biotin Nick End-Labeling (TUNEL) Assay
In Vivo
At 14 days after balloon injury, the intimal and medial areas of each cross section were measured and the ratio of intimal to medial area (I/M ratio) was calculated, as previously reported.17

We also measured the percentages of bromodeoxyuridine (BrdU)–positive cells, as previously reported,17 and the percentage of TUNEL-positive cells by using the Apoptag kit (Intergen) at 7 days after injury.

Aortic Vascular Smooth Muscle Cell Isolation
VSMCs were isolated from thoracic aortas of rats and mice using the collagenase digestion method and cultured as described.19 VSMCs were grown to 70% to 80% confluence and then made quiescent by incubation with DMEM containing 0.1% FCS for 48 hours before addition of serum.

Effects of ASK1 on Rat VSMC Proliferation and Migration In Vitro
In vitro gene transfer of Ad-E, DN-ASK1, or W-ASK1 to rat VSMCs was carried out by incubation with the adenoviral vector with multiplicity of infection of 200, as described.20 3H-thymidine incorporation and cell number after 2% serum stimulation were measured, as previously reported.19 Cell migration was measured with 48-well Boyden chamber method (Neuro Probe), as described.20

VSMC Proliferation and Migration From ASK1−/− and WT Mice In Vitro
DNA synthesis, cell number, and migration of VSMCs from WT or ASK1−/− mice with serum stimulation were measured in the same way as rat VSMCs.

Preparation of VSMC Protein Extracts and Western Blot Analysis
As previously reported,19 VSMCs from rats and mice were harvested by scraping with lysis buffer, sonicated, and centrifuged to obtain the supernatant. ASK1, p38, JNK, and ERK activities in VSMCs from

Figure 1. Time course of arterial ASK1 activity. Top, Representative Western blot analysis of phospho-ASK1 and α-tubulin at 0, 2, 5, and 15 minutes after balloon injury. Bottom, Level of arterial phospho-ASK1, corrected for α-tubulin, at each time point after injury. The mean value of phospho-ASK1 at 0 minutes is represented as 1. Each bar represents mean±SEM (n=4); *P<0.01 vs 0.

rats and mice were measured by Western blot analysis, as described above.

Mouse Femoral Injury
The surgical procedure for cuff-induced vascular injury in the femoral artery was performed, as previously described.21 At 28 days after injury, the I/M ratio was calculated, as described above.

Statistical Analysis
All data are presented as mean±SEM. Comparisons among groups were made by 1-way ANOVA, followed by Duncan multiple-range test. For differences between 2 groups, a Student’s t test was used when appropriate. Differences were considered statistically significant at a value of P<0.05.

Results
Time Course of Arterial ASK1 Activity After Balloon Injury
Arterial ASK1 activity was remarkably increased at 2 minutes after balloon injury, peaked at 5 minutes (7.0-fold increase compared with control), and returned to control level at 15 minutes (Figure 1).

Gene Transfer of DN-ASK1 and Effects on Signal Transduction
HA immunohistochemistry was performed on rat carotid artery subjected to DN-ASK1 or Ad-E gene transfer before 2 days. DN-ASK1 gene transfer produced the significant amount of the transgene expression all over the media (Figure 2A).

We examined the effect of DN-ASK1 on MAP kinase activities. Arterial MAP kinases containing p38, JNK, and ERK were remarkably activated at 5 minutes after balloon injury, being in good agreement with our previous report.17-19
The increase in JNK and p38 activities was significantly inhibited by gene transfer of DN-ASK1, but ERK activation was not affected (Figures 2B and 2C).

Effects of DN-ASK1 or W-ASK1 on Neointimal Formation After Injury
DN-ASK1 gene transfer significantly decreased the I/M ratio (0.57±0.06) at 14 days after balloon injury compared with Ad-E (1.04±0.06) (P<0.01; Figures 3A and 3B). Furthermore, W-ASK1 gene transfer significantly increased the I/M ratio (1.27±0.14) after injury. On the other hand, medial area was not different among Ad-E, DN-ASK1, and W-ASK1 gene transfer.

Effects of DN-ASK1 on VSMC Proliferation and Apoptosis In Vivo
The significant proliferation of intimal and medial cells was observed at 7 days after balloon injury. The BrdU index in the intima from the group transfected with DN-ASK1 was smaller than the group transfected with Ad-E (28.7±2.1% versus 44.6±1.7%; P<0.01). The BrdU index in the media from DN-ASK1 group was smaller than that from Ad-E–transfected group (4.1±0.1% versus 7.3±0.5%; P<0.01) (Figure 4A).

The significant apoptosis of intimal and medial cells was observed by balloon injury, being in good agreement with a previous report. However, the DN-ASK1 gene transfer did not significantly decrease the TUNEL index at 7 days after injury in both the intima and media (Figure 4B).

Time Course of ASK1 Activity and Effects of DN-ASK1 on MAP Kinase Activation of Rat VSMCs In Vitro
To determine whether ASK1 activation is directly concerned with VSMCs, we examined ASK1 activity in VSMCs in vitro and effects of DN-ASK1 on the activation of MAP kinases.
ASK1 activity in rat VSMCs was increased at 2 minutes after 2% serum stimulation and reached the peak by 3.3-fold at 5 minutes (Figure 5A). Stimulation of VSMCs with serum-activated ERK, p38, and JNK DN-ASK1 blocked serum-induced activation of p38 and JNK but did not change ERK activity (Figures 5B and 5C). Ad-E did not affect these activities at all.

Serum-Induced Rat VSMC Proliferation and Migration

We examined the role of ASK1 in VSMC proliferation and migration in vitro. As shown in Figure 6A, treatment of rat VSMCs with serum significantly increased the rate of DNA synthesis and cell number. Compared with Ad-E, DN-ASK1 inhibited serum-induced increase in 3H-thymidine incorporation by 40% and suppressed the increase in cell number by 33% (Figure 6A). Without serum stimulation, infection of Ad-E or DN-ASK1 did not significantly affect 3H-thymidine incorporation or cell number in VSMCs (data not shown).

As shown in Figure 6B, serum stimulation significantly induced VSMC migration. Infection of DN-ASK1 suppressed serum-induced VSMC migration by 33% (P<0.01). Ad-E did not significantly affect serum-induced cell migration. Compared with Ad-E, infection with W-ASK1 only slightly increased serum-induced 3H-thymidine incorporation, cell number, and migration in VSMCs.

ASK1 and MAP Kinase Activities, Proliferation, and Migration in VSMCs From ASK1−/− and WT Mice In Vitro

Tobiume et al11 have reported that ASK1−/− mice were indistinguishable in appearance from WT mice. Furthermore, there was no significant difference between ASK1−/− mice and WT mice in body weight, blood pressure, and left
ventricular weight at 10 weeks of age (unpublished data). Therefore, we compared vascular remodeling in ASK1−/− mice.

As shown in Figure 7A, ASK1 activity in VSMCs from WT mice was remarkably increased with serum stimulation. On the other hand, ASK1 activation was undetectable in VSMCs from ASK1−/− mice. Serum-induced p38 and JNK activation in VSMCs from ASK1−/− mice was less than that from WT mice, whereas serum-induced ERK activation in VSMCs from ASK1−/− mice was similar to that from WT mice.

To confirm the role of ASK1 in VSMC proliferation and migration, we isolated VSMCs from WT and ASK1−/− mice. A 3.1-fold increase in 3H-thymidine incorporation and a 2.3-fold increase in cell number were observed with WT VSMCs when they were treated with serum. On the other hand, a 2.1-fold increase in 3H-thymidine incorporation and an only 1.1-fold increase in cell number were observed in VSMCs from ASK1−/− mice. These increases in VSMCs from ASK1−/− mice were significantly smaller than VSMCs from WT mice (P<0.01) (Figure 7B). An in vitro assay for cell migration showed that WT VSMC migration was increased by 1.8-fold with serum stimulation. However, the migration of ASK1−/− VSMCs was significantly attenuated compared with WT VSMCs (P<0.01) (Figure 7C).

**Effects of ASK1 Deficiency on Neointimal Formation After Cuff Injury In Vivo**

To define the role of ASK1 in vascular hyperplasia, we compared the I/M ratio after cuff injury between WT and ASK1−/− mice. The I/M ratio at 28 days after cuff placement around the femoral artery was significantly smaller in ASK1−/− mice than in WT mice (0.26±0.05 versus 0.56±0.13, respectively) (P<0.05), whereas medial area was not different in each group (Figures 8A and 8B).

**Discussion**

To our knowledge, there is no available report on the roles of MAPKKKs, including ASK1, in vascular disease. The major findings of this work were that ASK1 was essential for neointimal formation after vascular injury and for VSMC proliferation in vitro and in vivo. Thus, ASK1 plays a critical role in vascular remodeling.

It is well known that MAP kinase signaling cascades are one of the most important signaling pathways responsible for cellular function. ASK1, initially identified as an apoptosis-inducing kinase, is activated in cells treated with various extracellular stimuli and relays those signals to the down-stream cascades. Very recently, it has been reported that...
ASK1 is implicated in various cellular functions. For example, Takeda et al.\textsuperscript{13} have reported that moderate expression of constitutively active ASK1 induces neuronal differentiation of undifferentiated PC12 cells and prolongs survival time of PC 12 cells under serum starvation, whereas excessive ASK1 activity induces apoptosis in PC12 cells. Sayama et al.\textsuperscript{14} have shown that expression of constitutively active ASK1 at a higher level strongly induces apoptosis in human keratinocytes. On the other hand, they have also shown that expression of constitutively active ASK1 at a lower level induces dramatic morphological changes with the induction of differentiation markers.\textsuperscript{14} Thus, although ASK1 may play broad and critical roles in a variety of cellular responses, the role of ASK1 in vitro depends on cell types or cellular context. Furthermore, there is no available report on the roles of MAPKKKs, including ASK, in vascular disease. These in vitro findings on the important role of ASK1 encouraged us to investigate the possible involvement of ASK1 in vascular disease in vivo.

In the present study, we first examined ASK1 activation in injured artery. Interestingly, arterial ASK1 was dramatically and transiently activated after balloon injury. Therefore, we investigated the significance of ASK1 in vascular diseases in vivo by direct blockade of ASK1 activation. To specifically inhibit the activation of vascular endogenous ASK1 in vivo, we transferred DN-ASK1 into rat carotid artery. To distinguish the transferred ASK1 mutant from the endogenous kinase, the mutants were HA-tagged. Immunohistochemistry with anti-HA antibody confirmed that DN-ASK1 was significantly expressed within the arterial cells (Figure 2A). Thus, our present method allowed us to elucidate the direct role of ASK1 in neointimal formation after injury. Of note are the observations that gene transfer of DN-ASK1 significantly prevented neointimal hyperplasia as shown by the I/M ratio and suppressed VSMC proliferation in either the intima or the media. To additionally validate that ASK1 participates in VSMCs proliferation in vascular hyperplasia, we compared in ASK1\textsuperscript{-/-} and WT mice neointimal formation after cuff placement around the femoral artery. The I/M ratio after cuff injury was significantly smaller in ASK1\textsuperscript{-/-} mice than in WT mice. These results provided the first evidence that ASK1 activation played the key role in the intimal thickening after vascular injury.

To explain the role of ASK1 in detail, we measured ASK1 activity in cultured VSMCs and also the effect of DN-ASK1 on proliferation and migration of cultured VSMCs. ASK1 activity was remarkably increased at 5 minutes after serum stimulation and remained increased until 15 minutes. DN-ASK1 inhibited serum-induced increase in VSMC proliferation. Migration of VSMCs, as well as proliferation, is the essential process for the formation of intimal hyperplasia in vascular disorder.\textsuperscript{3,24} Thus, it is an important question whether ASK1 directly participates in VSMC migration. DN-ASK1 inhibited serum-induced increase in VSMC migration. To additionally confirm the important role of ASK1 in VSMC proliferation and migration, we also examined VSMCs from ASK1\textsuperscript{-/-} mice. Both proliferation and migration of VSMCs isolated from ASK1\textsuperscript{-/-} mice were significantly attenuated compared with those from WT mice. These results showed that ASK1 plays a critical role in VSMC migration and proliferation. However, additional study is needed to elucidate whether the lesser proliferation and migration of VSMCs from ASK1\textsuperscript{-/-} mice can apply to the other types of cells.

Our present study did not permit us to determine the molecular mechanism responsible for ASK1-mediated vascular hyperplasia. Previous in vitro data show that ASK1 activates SEK1-JNK or MKK3/MKK6-p38 signaling cascades.\textsuperscript{9-11} However, the lower signaling cascades of ASK1 in vascular tissue in vivo remain to be determined. In this study, gene transfer of DN-ASK1 significantly suppressed the activation of either p38 or JNK after balloon injury but did not affect ERK activity. Furthermore, our present in vitro data showed that serum-induced p38 and JNK activation of cultured VSMCs, but not ERK activation, was significantly inhibited by DN-ASK1 and that serum-induced p38 and JNK
activation of VSMCs from ASK1−/− mice was lesser than that from WT mice. Thus, DN-ASK1 could inhibit both JNK and p38 activities after balloon injury. Taken together with our previous findings that either JNK or p38 participates in the development of intimal hyperplasia by balloon injury,17,19 p38 or JNK may be partially involved in ASK1-mediated vascular remodeling. However, additional study is needed to elucidate the precise mechanism underlying the inhibition of neointima by DN-ASK1.

Study Limitation

Previous reports showed that overexpression of ASK1 induces endothelial cell apoptosis in vitro.25 It is very interesting to investigate whether overexpression of ASK1 induces endothelial cell apoptosis in vivo. Unfortunately, balloon injury model is not suitable for examination of endothelial cell apoptosis, because balloon injury induces endothelial cell denudation, whereas it is well known to be very useful for estimation of the role of SMCs. Therefore, this model did not permit us to investigate the in vivo role of ASK1 in endothelial cell function in our present work.

In conclusion, we obtained the first evidence that ASK1 directly participates in VSMC proliferation and migration and neointimal thickening in injured artery. Thus, our present study provided a new insight into the molecular mechanism of neointimal hyperplasia. We propose that ASK1 seems to be a new therapeutic target for treatment of vascular diseases.

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