Important Role of Apoptosis Signal-Regulating Kinase 1 in Ischemia-Induced Angiogenesis

Yasukatsu Izumi, Shokei Kim-Mitsuyama, Minoru Yoshiyama, Takashi Omura, Masayuki Shiota, Atsushi Matsuzawa, Tokihito Yukimura, Toyoaki Murohara, Motohiro Takeya, Hidenori Ichijo, Junichi Yoshikawa, Hiroshi Iwao

Objective—We first examined the role of apoptosis signal-regulating kinase 1 (ASK1), one of mitogen-activated protein kinase kinase kinases, in ischemia-induced angiogenesis.

Methods and Results—Unilateral hindlimb ischemia was induced surgically in C57BL/6J wild-type (WT) mice or mice deficient in ASK1 (ASK1−/−). ASK1 activity in WT mouse hindlimb was increased dramatically after ischemia. By laser Doppler analysis, well-developed collateral vessels and angiogenesis were observed in WT mice in response to hindlimb ischemia, whereas these responses were reduced in ASK1−/− mice. Immunostaining revealed that infiltration of macrophages and T lymphocytes was suppressed in the ischemic tissues of ASK1−/− mice compared with WT mice. The expression of vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1) proteins in ischemic tissues was weaker in ASK1−/− mice compared with WT mice. In vitro study on endothelial cells indicated that dominant-negative ASK1 significantly attenuated hydrogen peroxide–induced VEGF and MCP-1 production. Furthermore, in vivo blockade of MCP-1 by its neutralizing antibody suppressed the recovery of the blood flow and capillary formation after ischemia.

Conclusions—ASK1 pathway promotes early angiogenesis by inducing inflammatory cell infiltration and VEGF and MCP-1 expression. ASK1 may provide the basis for the development of new therapeutic strategy for angiogenesis.

Key Words: angiogenesis • ischemia • inflammation • signal transduction • cytokines

Many investigators have delivered vascular endothelial growth factor (VEGF), basic fibroblast growth factor, or hepatocyte growth factor to induce angiogenesis.1,2 Iba et al3 have shown that implantation of mononuclear cells and mice.5 Thus, angiotensin II–AT1 receptor pathway is also involved in angiogenesis in vivo. However, the mechanism of intracellular signaling pathways in ischemia-induced angiogenesis remains unclear.

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 kinase (MAPK) cascades.6–8 ASK1 has been initially identified as an apoptosis-inducing kinase6,9 and has been reported recently to be implicated in a variety of cellular functions, including cell proliferation,10 survival,11 differentiation,12 and inflammatory response.13

In the present study, by using mice deficient in ASK1 (ASK1−/−), we investigated the role of ASK1 in angiogenesis, using a well-established mouse model of ischemia-induced angiogenesis. We obtained the first in vivo evidence that ASK1 plays a pivotal role in ischemia-induced angiogenesis.

Methods

Animals

All procedures were in accordance with institutional guidelines for animal research. Male ASK1−/− mice and WT (ASK1+/+) mice from the same genetic background (C57BL/6J) at the age of 8 to 10 weeks...
were used in the present study. C57BL/6J mice were obtained from Japan SLC (Shizuoka, Japan).

**Mouse Model of Angiogenesis**

Unilateral hindlimb ischemia was induced by resecting the left femoral arteries and veins of WT mice and ASK1−/− mice under anesthesia with sodium pentobarbital (50 mg/kg IP), as described previously.5,14

**Laser Doppler Blood Flow Analysis**

We measured hindlimb blood flow using a laser Doppler blood flow (LDBF) analyzer (Moor LDI; Moor Instruments) as described previously.5,14 Before and on postoperative days 1, 4, 7, 14, 21, and 28, we performed LDBF analysis over the legs and feet. After blood flow was scanned twice, the stored images were subjected to computer-assisted quantification, and the average flows of ischemic and nonischemic limbs were calculated. To avoid data variations attributable to ambient light and temperature, hindlimb blood flow was expressed as the ratio of left (ischemic) to right (nonischemic) LDBF.

**Angiographic Score**

On postoperative day 14, under pentobarbital anesthesia, the peritoneal cavity was opened, and a PE-10 catheter was inserted through the abdominal aorta. Angiography was then performed by injecting 0.3 mL of contrast media (lipiodol) through the catheter. X-ray angiograms were taken using a Microfocus X-Ray Television Device (Hitex Co Ltd), and the extent of collateral vessel formation was quantified by angiographic score as described previously.5,15

**Capillary Density**

Capillary density within the ischemic thigh adductor skeletal muscles was analyzed to obtain specific evidence of vascularity at the level of microcirculation. Three pieces of ischemic muscles were harvested from each animal, sliced, and fixed in methanol. Tissues were embedded in paraffin, and multiple tissue slices 5 μm in thickness were prepared. Capillary endothelial cells (ECs) were identified by immunohistochemical staining with a rat anti-mouse CD31 antibody (Ab; PharMingen). Fifteen random microscopic fields from 3 different sections in each tissue block were examined for the presence of capillary ECs, and capillary density was expressed as the number of capillaries per high-power field (×400).

**Histological Analysis of the Inflammatory Responses**

ASK1 has been shown to play an inflammatory role. Thus, we examined whether the extent of inflammatory reactions in the ischemic tissues differed between WT and ASK1−/− mice. Multiple sections, 5-μm thick, prepared from paraffin-embedded tissues of the ischemic limbs were used for histological analysis. Leukocyte infiltration was examined by hematoxylin and eosin (H&E) staining. Furthermore, infiltrated leukocytes, macrophages, and T lymphocytes were identified with anti-mouse CD45 Ab (PharMingen), respectively, and the number of positive cells was manually counted.

**VEGF and Monocyte Chemoattractant Protein-1 Expression in Ischemic Tissues**

We examined the expression of VEGF and monocyte chemoattractant protein-1 (MCP-1) in ischemic tissues. Cryostat sections, 15-μm thick, from frozen ischemic tissues were mounted on Matsunami adhesive silane (MAS)-coated slides. They were incubated with an anti-mouse VEGF Ab (Santa Cruz Biotechnology Inc.) or anti-mouse MCP-1 Ab (Santa Cruz Biotechnology Inc.) in a moist chamber overnight at 4°C. The slides were then incubated for 30 minutes at 37°C with Histofine secondary Ab (Nichirei Co.) to detect VEGF or MCP-1.

**Preparation of Extracts and Western Blot Analysis**

Our detailed method has been described previously.10,16 Protein extracts were obtained from homogenized ischemic or nonischemic skeletal muscles. After electrophoretic transfer, the membranes were probed with antibodies against phospho-ASK1, phospho-p38 MAPK (p38), phospho–c-Jun amino-terminal kinase (JNK) or phospho–extracellular signal-regulated kinase (ERK). All antibodies were purchased from New England Biolabs Inc. Furthermore, we evaluated the expression levels of VEGF and MCP-1 on days 3 and 7 after ischemia. VEGF Ab (P-20) and MCP-1 Ab (M-18; Santa Cruz Biotechnology Inc.) were used.

**Effects of MCP-1 Blockade on Ischemia-Induced Angiogenesis**

WT mice or ASK1−/− mice were injected intraperitoneally with 100 μg of mouse anti–MCP-1–neutralizing Ab17 or with 100 μg of normal mouse IgG (R & D Systems Inc.) immediately after induction of ischemia and postoperative days 1, 3, 5, and 7. We measured hindlimb blood flow until days 28 and capillary density on day 14, as mentioned above.

**Cell Culture, Adenovirus Gene Transfer, and Measurement of VEGF and MCP-1 Protein**

Human umbilical vein ECs (HUVECs) were purchased from Clonetics. Cells were cultured on 0.25% gelatin-coated dishes at 37°C, 5% CO₂ in endothelial basal medium (EBM; Clonetics) containing 5% FBS, 20 μg/mL EC growth supplement (Becton Dickinson), 50 U/mL penicillin, and 50 μg/mL streptomycin (Sigma Chemicals) and used between passages 3 and 5. They were infected with a recombinant adenovirus containing LacZ or dominant-negative mutant of ASK1 (DN-ASK1)18 at 50 multiplicities of infection in EBM for 1 hour, and then the medium was replaced with medium 199 containing 0.1% FBS. After starvation for 24 hours, cells were maintained in the culture medium containing 100 μmol/L hydrogen peroxide (H₂O₂). The cultured medium was exchanged every 24 hours for 3 days and collected. The concentration of MCP-1 and VEGF protein in the collected medium was quantified by using a commercial solid-phase ELISA (Pierce Biotechnology, Inc.).

**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 t test was used when appropriate. Differences were considered statistically significant at a value of P<0.05.

**Results**

**Time Course of ASK1 and MAPK Activation After Ischemia**

Tobiume et al18 have reported that ASK1−/− mice were indistinguishable in appearance from WT mice. Furthermore, there was not a significant difference between ASK1−/− mice and WT mice in body weight and blood pressure throughout the experimental period (Y.L., S.K.-M., unpublished data, 2004). Therefore, we compared in angiogenesis between ASK1−/− mice and WT mice.

All mice survived after surgical induction of unilateral hindlimb ischemia. We compared ASK1 and MAP kinase activities in ischemic hindlimb. As shown in Figure 1A, ASK1 in WT mice was remarkably activated at 2 minutes after hindlimb ischemia and peaked at 30 minutes (12-fold increase compared with control), whereas ASK1 was not detected in ASK1−/− mice.

As shown in Figure 1B and 1C, ischemia-induced p38 activation in ASK1−/− mice was lesser than that in WT mice.
JNK activity was increased slightly in WT mice after ischemia. On the other hand, ischemia-induced ERK activation in ASK1−/− mice was similar to that in WT mice.

Unilateral Hindlimb Ischemia and Time Course of LDBF Analysis
Immediately after the left femoral artery and vein were resected, the ratio of ischemic (left) to nonischemic (right) hindlimb LDBF (the LDBF ratio) decreased from 1.03±0.05 to 0.09±0.01 in WT and from 1.03±0.04 to 0.09±0.01 in ASK1−/− mice. Thus, the severity of induced ischemia was comparable in the 2 groups.

Figure 2A shows representative LDBF images of hindlimb blood flow. Serial LDBF examination disclosed progressive recovery of hindlimb blood flow in WT mice after induction of ischemia. On the other hand, the blood flow of ASK1−/− mice remained impaired during the follow-up period. Compared with WT mice, the LDBF ratio in ASK1−/− mice was persistently low after 7 days of induction of ischemia and significantly attenuated on day 28 (36% decrease; *P<0.01; Figure 2B).

Angiographic Score and Tissue Capillary Density
Next, we examined whether or not the reduced LDBF ratio was associated with the number of angiographically visible collateral vessels (arteriogenesis). On day 14, angiography revealed a smaller number of collateral vessels in the thigh adductor muscle area of ASK1−/− mice than WT mice (Figure 2C).

To investigate the extent of angiogenesis at the microcirculation level, we measured capillary density in histological sections harvested from the ischemic tissues. Quantitative analysis revealed that the capillary density was significantly reduced in ASK1−/− mice on postoperative day 14 compared with WT mice (Figure 2D).

Figure 1. Time course of ischemia-induced ASK1 and MAPK (p38, JNK, and ERK) activation in WT and ASK1−/− mice. A, Top panel shows representative Western blot analysis of ASK1 activation at 0, 2, 5, 15, 30, and 60 minutes after ischemia. Bottom panel shows the quantitative data at each time point. The mean value of ASK1 activation at 0 minutes is represented as 1. *P<0.01 vs 0. B, Representative Western blot analysis of MAPK activation at 0, 2, 5, 15, 30, and 60 minutes after ischemia. C, The quantitative data at each time point. The mean value of each MAPK at 0 minutes is represented as 1. Each bar represents mean±SEM (n=4 to 6). *P<0.01 vs control WT mice; ‡P<0.01 vs control ASK1−/− mice; #P<0.01. P-ASK1 indicates phospho-ASK1; P-p38, phospho-p38; P-JNK, phospho-JNK; P-ERK, phospho-ERK.

Figure 2. LDBF analysis, angiograms, and capillary density. A, Representative LDBF image. A low perfusion signal (dark blue) was observed in the ischemic hindlimb of an ASK1−/− mouse, whereas a high perfusion pattern (white to red) was detected in a control WT mouse. B, Computer-assisted quantitative analysis of hindlimb blood perfusion demonstrated a significant reduction in the ischemic/normal hindlimb blood flow ratio in ASK1−/− mice (n=11) compared with WT animals (n=13). Each bar represents mean±SEM. *P<0.01 vs WT at each time point. C, Representative angiograms (top) and angiographic score (bottom) on postoperative day 14 (n=6 in each group). D, Representative photomicrographs of tissue immunostained with anti-CD31 Ab (top) and capillary density (bottom) on postoperative day 14 (n=6 in each group). Each bar represents mean±SEM. *P<0.01 vs WT.
Inflammatory Responses

ASK1 plays inflammatory roles, and inflammation is an early trigger for ischemia-induced angiogenesis. Therefore, we examined histology of ischemic tissues on postoperative days 3 and 7. H&E staining revealed the infiltration of inflammatory cells in ischemic tissue of WT mice. In contrast, the number of infiltrated cells was smaller in the ischemic tissues of ASK1\(^{-/-}\) mice (Figure 3A).

To further determine the type of infiltrated cells, we stained tissue sections with common leukocyte marker (CD45), macrophage marker (F4/80 Ab), or T lymphocyte marker (CD3 Ab). The quantitative analysis revealed that the number of infiltrated leukocytes, macrophages, and T lymphocytes was lower in ASK1\(^{-/-}\) mice than in WT mice on day 3 (Figure 3B through 3D, respectively).

Expression of VEGF and MCP-1 in Ischemic Hindlimb

VEGF and MCP-1 are the major cytokines responsible for ischemia-induced angiogenesis as well as arteriogenesis. Therefore, we performed immunohistochemistry to examine the expression of VEGF and MCP-1 in the ischemic tissues of WT mice and ASK1\(^{-/-}\) mice. The expression of VEGF (Figure 4A) and MCP-1 (Figure 4B) proteins was weaker in ASK1\(^{-/-}\) mice than in WT mice on postoperative day 3.

Furthermore, we performed Western blot analysis to quantify VEGF and MCP-1 protein levels in the ischemic tissues of WT mice and ASK1\(^{-/-}\) mice. The expression of VEGF and MCP-1 proteins in ASK1\(^{-/-}\) mice was significantly reduced compared with WT mice on postoperative days 3 and 7 (Figure 4C).

Effects of DN-ASK1 on VEGF and MCP-1 Production in HUVECs

ECs release angiogenic cytokines including VEGF\(^{18,19}\) and MCP-1.\(^{20,21}\) To determine whether ASK1 activation is directly concerned with production of these cytokines, we examined the effect of DN-ASK1 on VEGF and MCP-1 protein in HUVECs. VEGF and MCP-1 protein was increased by H\(_2\)O\(_2\) stimulation. Gene transfer of DN-ASK1 significantly lessened H\(_2\)O\(_2\)-induced VEGF and MCP-1 production (Figure 5A and 5B). Treatment of LacZ did not affect their production at all.

In Vivo Blockade of MCP-1 Weakens Angiogenesis

We examined the effect of MCP-1 blockade on angiogenesis. Recovery of hindlimb blood flow in WT mice treated with anti–MCP-1 Ab is weaker than that in control IgG-treated WT mice after 14 days of ischemia induction (Figure 6A). The LDBF ratio in ASK1\(^{-/-}\) mice treated with anti–MCP-1 Ab was slightly but not significantly decreased compared with control ASK1\(^{-/-}\) mice (Figure 6A).

Figure 6B shows that the capillary density was reduced significantly in WT mice treated with anti–MCP-1 Ab com-
pared with those treated with normal IgG on postoperative day 14.

**Discussion**

The major findings in the present study are that angiogenesis, arteriogenesis, and blood flow recovery in response to hindlimb ischemia were impaired significantly in ASK1−/− mice compared with WT mice. Furthermore, we obtained the first evidence that ASK1 is directly involved in VEGF and MCP-1 expression. Thus, ASK1 is essential for ischemia-induced angiogenesis in vivo.

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,6,9 is activated in cells treated with various extracellular stimuli and relays those signals to the downstream cascades. Very recently, we and other investigators have reported that ASK1 is implicated in various cellular functions in vivo. We reported that ASK1 participates directly in neointimal hyperplasia in injured artery10 and that ASK1 is a critical signaling molecule responsible for angiogenesis II-induced cardiac hypertrophy, apoptosis, fibrosis, and coronary arterial remodeling.16 Furthermore, Yamaguchi et al have reported that ASK1 participates in left ventricular remodeling after myocardial infarction and transverse aortic constriction.22 Thus, ASK1 seems to play important roles in cardiovascular remodeling. However, there is no available report on the role of MAPKKKs, including ASK1, in angiogenesis in vivo. These data encouraged us to explore the potential role of ASK1 in ischemia-induced angiogenesis.

In this study, arteriogenesis such as angiographically visible collateral vessel formation was attenuated in ASK1−/− mice compared with WT mice. MCP-1, a C-C chemokine, is known to induce adhesion molecules and proinflammatory cytokines.23 Arteriogenesis as well as angiogenesis in response to tissue ischemia depends on macrophage infiltration,24,25 and MCP-1 recruits monocytes and macrophages. These findings suggest the possible involvement of MCP-1 in angiogenesis. However, the regulating mechanism of MCP-1 expression and the exact role of MCP-1 in ischemia-induced angiogenesis remain to be elucidated. Therefore, in this study, we examined MCP-1 expression in the ischemic hindlimb tissues of WT mice and ASK1−/− mice, by immunohistochemistry and Western blot analysis. We found the smaller expression of MCP-1 protein in ASK1−/− mice compared with WT mice after ischemia. To further confirm the important role of ASK1 in MCP-1 expression, we examined the effects of DN-ASK1 gene transfer on MCP-1 production in HUVECs in vitro. We found that DN-ASK1 significantly decreased H2O2-induced production of MCP-1 protein in HUVECs, thereby confirming that ASK1 directly regulates MCP-1 expression. Moreover, to elucidate whether the reduced expression of MCP-1 is directly linked to the impairment of angiogenesis in this ischemic model, we examined the effect of anti-MCP-1-neutralizing Ab on recovery of hindlimb blood flow after ischemia in mice. We obtained the evidence that anti-MCP-1 Ab significantly reduced recovery of hindlimb blood flow and formation of capillary density after ischemia. Thus, MCP-1 seems to play a critical role in ischemia-induced angiogenesis. All these results, together with the fact that infiltrated macrophages contribute to angiogenesis in this ischemia model,3 show that the impaired expression of MCP-1 in ASK1−/− mice, followed by the reduction of infiltration of macrophages, is responsible for the impairment of angiogenesis in ASK1−/− mice.

It has been established that VEGF promotes ischemia-induced and tumor-related angiogenesis.14,26–29 However, the molecular mechanism of VEGF expression in ischemic tissue is not fully understood. In the present study, immunohistochemistry and Western blot analysis indicated that endogenous expression of VEGF protein in ischemic tissues of ASK1−/− mice was reduced significantly compared with that of WT mice. Moreover, our in vitro experiments indicated that DN-ASK1 gene transfer significantly inhibited VEGF expression in H2O2-stimulated HUVECs. Thus, we obtained the evidence that ASK1 is involved in VEGF expression in ischemic tissue, which plays the critical role in angiogenesis.

Inflammation is an early key process for ischemia-induced angiogenesis and arteriogenesis.24 In the ischemic hindlimb, ASK1−/− mice exhibited smaller number of infiltrated polymorphonuclear leukocytes (PMNs), macrophages, or T lymphocytes than WT mice, showing that the lack of ASK1 leads to reduced inflammatory reactions in ischemic tissue. Iba et al9 showed that implantation of mononuclear cells and plate-
lets to ischemic limbs effectively induced collateral vessel formation, whereas implantation of PMNs failed it. Furthermore, it has been reported that not PMNs but infiltrated macrophages and T lymphocytes release VEGF, which promotes ischemia-induced and tumor-related angiogenesis.\textsuperscript{14,30} Therefore, the decreased infiltration of macrophages and T lymphocytes in ASK1\textsuperscript{−/−} mice, but not PMNs, contributes to the impaired angiogenesis after ischemia.

Our present study did not permit us to determine the molecular mechanism responsible for ASK1-mediated angiogenesis and arteriogenesis. In the present study, we first examined ASK1 activation in ischemic myocytes. Interestingly, ASK1 activity was dramatically increased after ischemia. It is well known that ischemia generates reactive oxygen species (ROS). Furthermore, previous work showed that ROS induces ASK1 activation in cardiac myocytes and neuronal cells.\textsuperscript{31,32} Therefore, ASK1 activation in ischemic skeletal myocytes might be mediated by ischemia-induced ROS. Previous data show that ASK1 activates SEK1-JNK or MKK3/MKK6–p38 signaling cascades.\textsuperscript{6,8,10,16} However, the lower signaling cascades of ASK1 in ischemic tissues in vivo remain to be determined. We showed that ischemia activated p38, JNK, and ERK of ischemic tissue. The absence of ASK1 activation in ASK1\textsuperscript{−/−} mice was accompanied by lesser activation of p38 and JNK than WT mice after ischemia.

Together with a previous report that p38 enhances tissue repair in ischemic diseases,\textsuperscript{33} it is conceivable that p38 may be at least partially involved in ASK1-mediated arteriogenesis. However, further study is needed to elucidate the precise mechanism underlying ASK1-mediated angiogenesis and arteriogenesis.

In conclusion, our present work provided the first in vivo evidence that ASK1 is activated by ischemia and plays a key role in ischemia-induced angiogenesis by inducing VEGF and MCP-1 expression. Thus, ASK1 is a critical signaling molecule responsible for ischemia-induced angiogenesis. We propose that ASK1 seems to be a new therapeutic target for vascular diseases.

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