Neurobiology

Role of Apoptosis Signal-Regulating Kinase 1 in Stress-Induced Neural Cell Apoptosis *in Vivo*

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Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase kinase kinase that plays an important role in oxidative stress-induced apoptosis. In the present study, we used ASK1 knockout (KO) mice to examine the possibility that ASK1 is involved in the neural cell apoptosis that occurs during retinal development and ischemic injury. ASK1 was expressed in retinal neurons, including retinal ganglion cells (RGCs), but retinal structure and extent of cell death during development were normal in ASK1 KO mice. On the other hand, the strain was less susceptible to ischemic injury, and the number of surviving retinal neurons was significantly increased compared with that in wild-type mice. Interestingly, ischemia-induced phosphorylation of p38 mitogen-activated protein kinase (p38), which mediates RGC apoptosis, was almost completely suppressed in ASK1 KO mice. In such retinas, the numbers of cleaved caspase-3- and

TUNEL-positive neurons were apparently decreased compared with those in wild-type mice. Furthermore, cultured RGCs from ASK1 KO mice were resistant to H₂O₂-induced apoptosis. Our findings suggest that ASK1 is involved in the neural cell apoptosis after various kinds of oxidative stress. Thus, inhibition of the ASK1-p38 pathway could be useful for the treatment of neurodegenerative diseases including glaucoma. (*Am J Pathol* 2006, 168:261–269; DOI: 10.2353/ajpath.2006.050765)

Ischemic retinal injury is implicated in a number of pathological states, such as retinal artery occlusion, glaucoma, and diabetic retinopathy.^{1–4} Thus, it is necessary to understand the molecular regulation after ischemic injury. It has been proposed that free radicals are important mediators of damage caused by retinal ischemia.^{5,6} Reperfusion injury after ischemia appears paradoxical, but oxygen-derived and other free radicals are principally formed when reduced compounds that accumulate during ischemia are reoxidized.³ There is evidence that this free radical burst, produced during the early stage of reperfusion, overwhelms normal cellular antioxidant defense mechanisms, causing oxidative stress and a variety of types of tissue injury.^{7,8} However, the detailed molecular mechanism of oxidative stress-induced neural cell apoptosis remains to be elucidated.

Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase (MAPK) kinase kinase, which is activated in response to various stimuli through distinct mechanisms.⁹ Overexpression of wild-type or constitutively active ASK1 induces apoptosis in various cell types through mitochondria-dependent caspase activation.^{10,11} On the other hand, oxidative stress and tu-

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mor necrosis factor-induced apoptosis are suppressed in ASK1-deficient cells.¹² Recent studies have shown that ASK1 relays its apoptotic signals to the stress-activated MAPK family members, c-Jun N-terminal kinase (JNK) and p38 MAPK (p38).¹³ p38 is activated and phosphorylated by environmental stress such as H₂O₂ and UV-B radiation, as well as proinflammatory cytokines like interleukin-1 and tumor necrosis factor.¹⁴ Lipton and co-workers^{15,16} previously reported that axotomy of the optic nerve or intraocular injection of *N*-methyl-D-aspartate activates p38, which leads to neural cell apoptosis, especially for retinal ganglion cells (RGCs). These results suggest the possibility that the ASK1-p38 pathway induces retinal death in various pathological conditions. In the present study, we attempted to elucidate a role of ASK1 during retinal development and ischemic injury by analyzing ASK1 knockout (KO) mice. We provide evidence for a critical role of the ASK1-dependent cell death pathway in the retina.

Materials and Methods

Animals

Experiments were performed using ASK1 KO mice¹² and their littermates in accordance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Vision Research. Light intensity inside the cages ranged from 100 to 200 lux under a 12-hour light/dark cycle.

Histological Analysis

For developmental studies, mice were sacrificed at embryonic day 15 (E15), E17, postnatal day 0 (P0), P7, P14, and P90. For E15 and E17 mice, their heads were fixed in 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffer (PB; pH 7.4) containing 0.5% picric acid for 2 hours at 4°C and embedded in paraffin wax. They were sectioned transversely at 7-μm thickness, mounted, and stained with hematoxylin and eosin. For P0, P7, P14, and P90 mice, they were anesthetized with diethylether and perfused transcardially with saline, followed by 4% PFA in 0.1 mol/L PB containing 0.5% picric acid at room temperature. The eyes were removed and postfixed in the same fixative for 2 hours at 4°C and embedded in paraffin wax. The posterior part of the eyes was sectioned sagittally at 7-μm thickness, mounted, and stained with hematoxylin and eosin.

In Situ Hybridization

Sense and antisense cRNA probes corresponding to 431 bases (nucleotides 283 to 713) of the human *Ask1* cDNA (GenBank accession no. D84476) were generated. This region is contained in the first coding exon of human *Ask1* gene, which is deleted in ASK1 KO mice.¹² Nucleotides 283 to 713 of the human *Ask1* cDNA were amplified by PCR using human ASK1 HA cDNA in pcDNA3 as template. The following primers were designed to amplify this

region: sense primer, 5'-GACGAGGGCATCACTTTCTC-3'; and antisense primer, 5'-GCGTTGTAAAAACGGTC-CAG-3'. The PCR products were subcloned into pGEM-T easy Vector (Promega, Madison, WI) according to manufacturer's protocol. Probes were synthesized from this cDNA template with SP6 (sense) or T7 (anti-sense) RNA polymerases (Roche, Basel, Switzerland) and digoxigenin-RNA labeling mix (Roche).

Mice were perfused transcardially with ice-cold 0.1 mol/L phosphate buffered saline (PBS; pH 7.4) and then with 4% PFA in 0.07 mol/L PB. Eyes were immediately enucleated and immersed in the same fixative for 2 hours at 4°C, followed by immersion in a sucrose solution (30% in PB) overnight at 4°C. Eyes were embedded in optimal cutting temperature compound (Tissue-Tek, Tokyo, Japan) and frozen on dry ice. The posterior parts of the eyes were sectioned at 10-μm thickness, collected on MAS-coated slides (Matsunami, Osaka, Japan), and processed as previously reported.^{17,18} Briefly, the sections were washed with PBS and treated with 2 μg/ml Proteinase K for 30 minutes at 37°C. They were fixed with 4% PFA in PB for 10 minutes, treated with 0.2 mol/L HCl for 10 minutes, and then treated with 0.25% acetic anhydride in 0.1 mol/L triethanol amine for 10 minutes. Hybridization was performed overnight at 50°C with 500 ng/ml probe in 3× standard saline citrate, 50% formamide, 0.12 mol/L PB, 1× Denhardt's solution, 125 μg/ml tRNA, 100 μg/ml salmon sperm DNA, and 10% dextran sulfate. After washing probe, the sections were incubated with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche) diluted 1:1000 in 0.1 mol/L Tris-HCl buffer [pH 7.4] containing 0.15 mol/L NaCl and 0.01% Tween-20. After washing, visible signal was detected with 5-bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride substrate system (DAKO, Carpinteria, CA).

Immunohistochemistry

Sections were prepared as described for histological analysis section. They were treated with 0.3% H₂O₂ for blocking endogenous peroxidase. After washing in PBS, they were blocked with PBS containing 0.1% normal horse serum and 0.4% Triton-X 100 for 1 hour at room temperature. They were incubated overnight at 4°C with a rabbit polyclonal antibody against active caspase-3 (1:10,000; R&D Systems, Minneapolis, MN), active p38 (1:100; Promega), a goat polyclonal antibody against Brn3b (1.0 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), a mouse monoclonal antibody against calretinin (1.0 μg/ml; Chemicon, Temecula, CA), calbindin (2.0 μg/ml; Sigma, Saint Louis, MO), protein kinase C (2.0 μg/ml; Sigma), glutamine synthetase (1.0 μg/ml; Chemicon), and glial fibrillary acidic protein (5.0 μg/ml; Progen, Heidelberg, Germany). The sections were then incubated with goat anti-rabbit or anti-mouse immunoglobulins conjugated to peroxidase labeled-dextran polymer (DAKO EnVision; DAKO), or biotinylated rabbit anti-goat IgG (Nichirei, Tokyo, Japan) and peroxidase-conjugated streptavidin (Nichirei) for 30 minutes and visualized with diaminobenzidine substrate kit (DAKO).

Ischemic Retinal Injury

Ischemia was achieved, and the animals were treated essentially as previously described.^{1,19–21} Briefly, we instilled sterile saline into the anterior chamber of the left eye at 120 cm of H₂O pressure for 20 minutes while the right eye served as a nonischemic control. Seven days after reperfusion, animals were sacrificed, and the posterior part of the eyes was sectioned sagittally at 7- μ m thickness through the optic nerve, mounted, and stained with hematoxylin and eosin. Ischemic damage was quantified in three ways. First, the thickness of the inner retinal layer (IRL; between the internal limiting membrane and the interface of the outer plexiform layer and the outer nuclear layer) was measured. Second, in the same sections, the number of neurons in the ganglion cell layer (GCL) was counted from one ora serrata through the optic nerve to the other ora serrata. Third, RGCs were retrogradely labeled from the superior colliculus (SC) with Fluoro-Gold (FG; Fluorochrome, Englewood, CO)^{15,22} soon after ischemic injury. Each midbrain was exposed, and after removing the skull using a micro drill, 2 μ l of 4% FG was injected into the SC to mark the RGCs by retrograde axonal transport. Seven days after FG application, the eyes were enucleated and the retinas were detached and prepared as flattened whole mounts in 4% PFA in 0.1 mol/L PBS solution. GCL was examined in whole-mounted retinas with fluorescence microscopy to determine the RGC density. Four standard areas (0.04 mm²) of each retina at the point of 0.1 mm from the optic disk were randomly chosen, labeled cells were counted by observers blinded to the identity of the mice, and the average number of RGCs per square millimeter was calculated. For statistical analysis, six animals were used for each wild-type (WT) and ASK1 KO group. The changes in RGC number after ischemia were expressed as a percentage of the WT control eyes.

Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) Staining

Paraffin sections were treated with 10 μ g/ml Proteinase K and then incubated in 0.26 U/ μ l terminal deoxynucleotidyl transferase in the supplied 1 $\times$ buffer (Invitrogen, Carlsbad, CA) and 20 μ mol/L biotinylated-16-dUTP (Roche) for 1 hour at 37°C. The sections were washed three times in PBS and blocked for 30 minutes with 10% normal donkey serum in PBS. The sections were then incubated with dichlorotriazinyl amino fluorescein-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) and diluted 1:500 in PBS for 30 minutes.

Quantification of Retinal Cell Apoptosis

Cell death was quantified by an ELISA (Roche) using a combination of antibodies recognizing histones and DNA, allowing the quantification of soluble nucleosomes in cell lysates.²³ A mouse retina was homogenized in 100 μ l of PBS containing 1 mmol/L phenylmethylsulfonyl fluoride and centrifuged at 15,000 $\times g$ for 10 minutes. A

portion of the supernatant was used to quantify protein concentration, and the rest was processed. Absorbance values ranged between 0.1 and 1.0 and were normalized with respect to the values obtained with control retinas.

Immunoblot

Whole retinas were quickly removed at 3, 6, or 24 hours after ischemic injury. Retinas were homogenized in 200 μ l of ice-cold 50 mmol/L Tris-HCl [pH 7.4] containing 150 mmol/L NaCl, 10 mmol/L NaF, 1 mmol/L Na₃VO₄, and the protease inhibitor cocktail (Roche). The protein concentration of the samples was determined by the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Three micrograms of the protein were separated on an 11% sodium dodecyl sulfate-polyacrylamide gel and subsequently transferred to an Immobilon-P filter (Millipore, Billerica, MA). Membranes were incubated with anti-p38 α (1:1000; Cell Signaling, Beverly, MA) or anti-phospho-p38 α monoclonal antibody (1:1000; Cell Signaling). They were then incubated with a horseradish peroxidase-labeled anti-mouse IgG antibody (Amersham Bioscience, Piscataway, NJ) and visualized using an ECL Western blotting system (Amersham Bioscience).

Isolation of RGCs

RGCs were isolated by using a previously described two-step panning method.^{24–26} Briefly, P6 mice were killed to obtain approximately 20 enucleated eyes for each experiment. Isolated retinas were incubated in calcium- and magnesium-free Hanks' balanced salt solution containing 0.5 mg/ml papain, 0.24 mg/ml cysteine, and 0.5 mmol/L EDTA for 10 minutes, and dissociated cells were incubated with rabbit anti-mouse macrophage antibody (Inter-Cell Technologies, Hopewell, NJ) for 5 minutes. Cell suspensions were treated for 30 minutes in 100-mm petri dishes coated with goat antibody against rabbit IgG heavy and light chains (Southern Biotechnology Associates, Birmingham, AL). Suspensions containing the cells that did not adhere to the petri dish were treated for 1 hour in 100-mm petri dishes coated with anti-mouse Thy 1.2 antibody (Serotec, Oxford, UK). Cells that adhered to the second petri dish were collected after separation by a 5-minute incubation with 0.05% trypsin and 0.53 mmol/L EDTA and were incubated in 96-well plates for cell death assay or in 8-well chamber slides for identifying the purity of isolated RGCs.

Culture plates or chamber slides were coated with 0.1 mg/ml poly-D-lysine (Sigma, St. Louis, MO) for at least 4 hours. They were then additionally coated overnight with 5 μ g/ml Engelbreth-Holm-Swarm-laminin (Sigma). Medium developed by Politi et al²⁷ for monolayer culture of mixed mouse retinal neurons, as modified for use in this experiment, consisted of Dulbecco's modified Eagle's medium with the additions of 1.6 μ mol/L insulin, 40 nmol/L progesterone, 60 nmol/L selenite, 125 nmol/L transferrin, 200 μ mol/L putrescine, 100 nmol/L hydrocortisone, 5.2 μ mol/L cytidine-5'-diphosphocholine, 2.9 μ mol/L cytidine-5'-diphosphoethanolamine, 80 ng/ml

each of brain-derived neurotrophic factor and ciliary neurotrophic factor, 10 μ mol/L forskolin, and 1% fetal bovine serum. Cells were incubated at 37°C in humidified 5% CO₂ and 95% air.

Identification of Isolated RGCs

Mice were anesthetized by hypothermia, and RGCs were retrogradely labeled with 2 μ l of 4% FG at P3. After 3 days, RGCs were purified and seeded in culture as described in the former section. In some experiments, we prepared whole-mounted retinas from FG-injected mice and determined whether injected FGs are really transported into the RGCs in a retrograde manner. FG-labeled RGCs were observed to be uniformly distributed in retinal tissue (data not shown). Three days after preparation, cultured RGCs were fixed with 10% formalin in PBS labeled with propidium iodide and assessed for double-labeling with FG.

Assessment of H₂O₂-Induced Cell Death in Cultured RGCs

WT and ASK1 KO RGCs were seeded at density of 5×10^4 cells/well and cultured with 0.1 ml of medium on a 96-well culture plate. After 2 days, they were unstimulated (control), or stimulated with 2.5 mmol/L H₂O₂ for 16 hours. After incubation, 50 μ l of culture medium was collected and analyzed using a lactate dehydrogenase (LDH) cytotoxic test kit (Wako, Osaka, Japan), to estimate cell damage. LDH activity after H₂O₂ stimulation was normalized by subtracting control values from unstimulated cells.

Statistics

Data are presented as means $\pm$ SD except as noted. When statistical analyses are performed, Student's *t*-test or Mann-Whitney's *U*-test were used to estimate the significance of the results. Statistical significance was accepted at *P* < 0.05.

Results

ASK1 Expression during Retinal Development

We first examined ASK1 mRNA expression during mouse retinal development by *in situ* hybridization analysis. ASK1 expression was obscure at E15 (Figure 1A) and E17 (data not shown) but clearly visible in the retinal GCL, which is still composed of multiple layers, at P0 (Figure 1B). In postnatal mice, ASK1 expression was observed in the mature GCL and weakly in the inner nuclear layer (INL) (Figure 1, C and D). In contrast, we did not detect staining in retinal sections from WT mice treated with control sense probe (Figure 1E) or those from ASK1 KO mice treated with antisense probe (Figure 1F). These results demonstrate that ASK1 is expressed in retinal

Figure 1. Expression of ASK1 during retinal development. **A–F:** *In situ* hybridization analysis of wild-type (**A–E**) and ASK1 KO (**F**) mice retina at E15 (**A**), P0 (**B**), P7 (**C**), and P90 (**D–F**). ASK1 expression was obscure at E15 (**A**) but appeared in the inner retina after P0 (**B–D**). Retinal sections from wild-type mice incubated with control sense probe (**E**) and those from ASK1 KO mice incubated with antisense probe (**F**) did not show staining. NBL, neuroblast layer; INL, inner nuclear layer. Scale bar = 50 μ m.

neurons including RGCs after birth and suggest that ASK1 may be involved in postnatal retinal development.

Effect of ASK1 on Programmed Cell Death and Retinal Morphology during Development

Because ASK1 may be involved in programmed cell death (PCD) in the developing retina, we next examined whether the development of the retina is perturbed in ASK1 KO mice. Figure 2 illustrates the retinal development of ASK1 KO and their WT littermates. The time course of retinal development in ASK1 KO mice (Figure 2, B, D, and F) was normal compared with that in WT mice (Figure 2, A, C, and E). In addition, retinal thickness and construction in adult mice (Figure 3A) were also normal in ASK1 KO mice (Figure 3B). We further examined WT and ASK1 KO mouse retinas with various cell type-specific markers (data not shown). The expression pattern of Brn3b (ganglion cells), calretinin (ganglion and amacrine cells), calbindin (horizontal cells), protein kinase C (bipolar cells), glutamine synthetase (Müller glial cells), and glial fibrillary acidic protein (astrocytes) showed no remarkable change between WT and ASK1 KO mice. We next quantified PCD in the whole retina at E15, E17, P0, and P7 using an ELISA method.²³ Consistent with the normal retinal development, extent of retinal cell death in the developing retina was normal in ASK1 KO mice (data not shown). Thus, the absence of ASK1 signaling did not seem to be involved in PCD, differentiation, or migration of retinal cells or their assembly into defined layers.

Figure 2. Retinal development in ASK1 KO mice. **A–F:** Hematoxylin and eosin staining of retinal sections at E15 (**A** and **B**), P0 (**C** and **D**), and P14 (**E** and **F**) in WT (**A**, **C**, and **E**) and ASK1 KO (**B**, **D**, and **F**) littermates. Asterisks in **A** and **B** indicate lens. NBL, neuroblast layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar = 50 μ m.

Effect of ASK1 on Ischemia-Induced Neural Cell Death

Because ASK1 may mediate stress-induced neural cell apoptosis in adult retina, we next examined the effect of ischemic injury, which is severe oxidative stress,^{3,20} in WT and ASK1 KO mice. Histological evaluation demonstrated a significant decrease in ischemic damage in ASK1 KO mice (Figure 3D) compared with their WT littermates (Figure 3C). The thickness of the IRL after ischemia was decreased to $65 \pm 2\%$ ($n = 6$) in WT, but

Figure 3. Quantitative analysis of ischemic retinal injury in ASK1 KO mice. **A–D:** Hematoxylin and eosin staining of control (**A** and **B**) and ischemic (**C** and **D**) retinal sections from WT (**A** and **C**) and ASK1 KO (**B** and **D**) mice. **E** and **F:** Thickness of the IRL (**E**) and the number of cells in the GCL (**F**) before and after ischemic injury as a percentage of the WT control eyes. Results of six independent experiments are presented as means $\pm$ SD. Note the decreased neural cell loss in ASK1 KO mice. INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar = 50 μ m.

$81 \pm 2\%$ ($n = 6$) in ASK1 KO mice (Figure 3E). Second, the percentage of surviving cells in the GCL was also increased in ASK1 KO ($81 \pm 1\%$; $n = 6$) compared with WT mice ($71 \pm 3\%$; $n = 6$) (Figure 3F). These results suggest that inner retinal neurons are less susceptible to ischemic injury in ASK1 KO mice. Because GCL may contain RGCs, displaced amacrine cells, and other minor cell types,²⁸ we next performed retrograde labeling of RGCs with FG and tried to determine the effect of ASK1 on RGC survival. Figure 4, A–H, shows the representative results of RGC labeling in ASK1 KO mice and their WT littermates. Consistent with the results of GCL counting (Figure 3F), the extent of ischemia-induced RGC death seemed to be mild in ASK1 KO mice (Figure 4, D and H) compared with their littermates (Figure 4, C and G, respectively). Quantitative analysis revealed that FG-labeled (surviving) RGCs after ischemia were significantly increased in ASK1 KO mice ($82 \pm 2\%$; $n = 6$) compared with their WT littermates ($65 \pm 1\%$; $n = 6$) (Figure 4I). These results demonstrate that loss of endogenous ASK1 has a protective effect on inner retinal neurons, especially for RGCs, after ischemic injury.

Involvement of ASK1 Signaling in Ischemia-Induced Neuronal Apoptosis

We next analyzed apoptotic cells in the retina by TUNEL staining 1 day after ischemic injury. Control animals showed practically no signals in both WT and ASK1 KO mice (data not shown). In ischemic retinas, many TUNEL-positive cells were observed in the inner retina in WT (Figure 5A) but not in ASK1 KO mice (Figure 5B). We previously demonstrated that ischemia-induced retinal cell apoptosis in the inner retina is executed mainly by caspase-3.²⁰ In fact, immunohistochemical analysis showed many cleaved caspase-3-positive cells in the inner retina in WT mice (Figure 5C), but the number was apparently decreased in ASK1 KO mice (Figure 5D). Previous studies have suggested a possibility that ASK1 could activate p38 MAPK pathway, leading to oxidative stress-induced RGC apoptosis.^{13,15,16} To determine this possibility, we examined total and phosphorylated/activated p38 α immunoreactivity after ischemic injury in WT and ASK1 KO mice. Immunoblot analysis revealed that the levels of phosphorylated p38 α in nonischemic control retinas were very low in both strains (Figure 6A, top panel). However, ischemic injury strongly induced phosphorylation of p38 α at 3, 6, and 24 hours after ischemic injury in WT retina (Figure 6B). On the other hand, phosphorylated p38 α immunoreactivity at 3 hours was low and essentially disappeared by 24 hours after ischemia in ASK1 KO mice (Figure 6, A and B). Nonetheless, total p38 α immunoreactivity (including both phosphorylated and nonphosphorylated forms) did not change after ischemia in both WT and ASK1 KO mice (Figure 6A, bottom panel). We also examined the localization of phosphorylated p38 α immunoreactivity in the mouse retina. In control retinas, phosphorylated p38 α -positive cells were absent (data not shown). In contrast, 3 hours after ischemia,

Figure 4. Quantitative analysis of retinal ganglion cell number in ASK1 KO mice. **A–H:** Retrograde labeling of retinal ganglion cells (RGCs) 7 days after ischemic injury (**C, D, G, and H**) and nonischemic fellow eyes (**A, B, E, and F**, respectively) in WT (**A, C, E, and G**) and ASK1 KO (**B, D, F, and H**) mice. **E** through **H** are enlarged images of **A** through **D** at approximately the same distance (0.1 mm) from the optic disk (eg, asterisk in **A**), respectively. **I:** RGC number before and after ischemic injury as a percentage of the WT control eyes. The number of labeled RGCs within four fields of identical size (0.04 mm²) at approximately the same distance (0.1 mm) from the optic disk were counted, and RGCs/mm² were calculated. Results of six independent animals are presented as means $\pm$ SD. Note the increased RGC resistance against ischemic injury in ASK1 KO mice. Scale bar = 500 μ m in **A** through **D** and 100 μ m in **E** through **H**.

Figure 5. Localization of apoptotic cells, cleaved caspase-3, and phosphorylated p38 immunoreactivity in ischemic retina. **A** and **B:** Representative pictures of TUNEL staining in WT (**A**) and ASK1 KO (**B**) mice retina. **C–F:** Immunohistochemical analysis of cleaved caspase-3 (**C** and **D**) and phosphorylated p38 α (**E** and **F**) 3 hours after ischemic injury. INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar = 90 μ m in **A** and **B** and 50 μ m in **C** through **F**.

phosphorylated p38 α -like immunoreactivity was observed in the inner retina in WT (Figure 5E) but almost absent in ASK1 KO (Figure 5F) mice. These results suggest that ischemic injury induces neural cell apoptosis, especially for RGCs, through the activation of p38 MAPK and caspase-3 under the regulation of ASK1.

Involvement of ASK1 in RGC Apoptosis

We next assessed the requirement of ASK1 for oxidative stress-induced apoptosis by using cultured RGCs from ASK1 KO mice. To determine the purity of our culture cells, mouse RGCs were retrogradely labeled with FG at P3, and culture cells were prepared at P6. Three days after preparation, these cultured cells were labeled with propidium iodide (PI) and assessed for double-labeling with FG. As shown in Figure 7, most cultured cells ($89 \pm 1\%$; $n = 4$) were double-labeled (yellow in Figure 7C) with FG (green in Figure 7A) and propidium iodide (red in Figure 7B). By using such relatively pure cultured RGCs, we examined the effect of H₂O₂ on RGC death. H₂O₂-induced apoptosis was clear in WT RGCs, as determined by examining extracellular LDH activities (Figure 7D). On the other hand, RGCs from ASK1 KO mice were more resistant to H₂O₂ compared with WT RGCs (Figure 7D). These results clearly indicate a role of ASK1 in RGC apoptosis under stress conditions.

Figure 6. Immunoblot analysis of p38 α immunoreactivity in ischemic retina. **A:** Three micrograms of retinal protein were prepared from ischemic eyes and nontreated fellow eyes and separated in 11% sodium dodecyl sulfate-polyacrylamide gel followed by immunoblot analysis using anti-p38 α and anti-phospho-p38 α antibodies. Representative image of three independent experiments are shown. **B:** Quantitative analysis of phosphorylated p38 α immunoreactivity at 3, 6, and 24 hours after ischemic injury. Results of three independent experiments are presented as means $\pm$ SD. Note the decreased phosphorylation of p38 α in ASK1 KO mice. * $P < 0.01$.

Discussion

In the present study, we demonstrated that the ASK1 MAPK kinase signaling pathway is involved in neural cell apoptosis after ischemic retinal injury *in vivo* (Figures 3 and 4). In addition, we found that both p38 MAPK and caspase-3 activation are clearly suppressed in ASK1 KO mice (Figures 5 and 6). Our results are consistent with previous studies describing that activation of p38 induces RGC apoptosis after axotomy of the optic nerve or mediated by glutamate neurotoxicity.^{15,16} Ischemic injury is associated with excessive concentrations of glutamate, which results in overactivation of glutamate receptors and initiates a cascade of events that leads to necrosis and/or apoptosis. Consistently, selective inhibition of glutamate receptor antagonists^{2,3} or *N*-acetylated- α -linked-acidic dipeptidase, an enzyme responsible for the hydrolysis of neuropeptide *N*-acetyl-aspartyl-glutamate to *N*-acetyl-aspartate and glutamate, protects neurons in animal models of stroke and ischemic retinal injury.^{19,29} Furthermore, we previously showed that glutamate transporter KO mice are susceptible to ischemic retinal injury.¹ Therefore, as well as p38 inhibition, appropriate control of glutamate concentration and/or glutamate receptor activation could be useful against ischemic injury.^{3,30}

Figure 7. Quantitative analysis of H₂O₂-induced RGC death in ASK1 KO mice *in vitro*. **A–C:** Cultured RGCs were prepared from mouse retinas that had been retrogradely labeled with FG. Most cultured cells (89 $\pm$ 1%, $n = 4$) were double labeled (yellow in **C**) with FG (green in **A**) and propidium iodide (PI; red in **B**), thus confirming the purity of our cultured RGCs. Scale bar = 100 μ m. **D:** Extent of RGC death after stimulation with 2.5 mmol/L H₂O₂ was quantified by examining extracellular LDH activities. Note the decrease of H₂O₂-induced death in RGCs from ASK1 KO mice. Results of three independent experiments are presented as means $\pm$ SD. * $P < 0.05$.

In addition to typical glutamate neurotoxicity, reactive oxygen species, such as superoxide and H₂O₂, are important mediators in damage caused by retinal ischemia.^{5,6} Our present study demonstrated that H₂O₂-induced RGC death is attenuated in ASK1 KO mice *in vitro* (Figure 7). Under stress conditions, several stress proteins, such as ubiquitin, play important roles as heat shock- and stress-regulated proteins.³¹ However, recent studies have shown that ubiquitin promotes either cell survival or apoptosis.^{32–35} In neural cells, excessive ubiquitin induction after ischemic injury may rather lead to neural cell apoptosis by the degradation of antiapoptotic proteins and suppression of the transcription of prosurvival proteins.²⁰ These results suggest a possibility that modulation of stress-responsive systems can become a “double-edged sword.” Although our present results suggest its usefulness, further investigations revealing the precise effect of complete ASK1 inhibition *in vivo* will be needed. On the other hand, ASK1 activity and consequent activation of downstream signaling molecules can be negatively regulated by stimulating phosphoinositide-3 kinase-Akt pathway. It was reported that Akt-mediated phosphorylation of ASK1 inhibits its ability to activate JNK/p38 and protects cells against H₂O₂-induced apoptosis.³⁶ Another possible factor that negatively regulates ASK1 activity is thioredoxin (Trx).³⁷ In resting cells, ASK1 constantly forms an inactive complex with Trx, whereas on stimulation, ASK1 is dissociated from Trx and activated by conformational changes and covalent modifications. Recent studies have suggested the possibility that Trx is a kind of neurotrophic factor released from retinal pigmented epithelial cells and plays a crucial role in maintaining photoreceptors from light damage.³⁸ Because other trophic factors such as ciliary neurotrophic factor and glial cell line-derived neurotrophic factor supplied from Müller glial cells rescue neural cells,^{4,23,39,40} application of Trx, in combination with these trophic factors, may stimulate multiple cellular targets and activate

separate mechanisms to rescue RGCs as well as photoreceptors during retinal degeneration. Further studies determining potential mechanisms other than ASK1-p38 pathway, which may be involved in the regulation of ischemia-induced neural cell death, will be needed.

In contrast to the dramatic alternation in adult neuronal apoptosis, extent of PCD, developmental process, structure, and cell type-specific population were all normal in ASK1 KO retina (Figure 2). There are two periods of cell death in the developing retina.⁴¹ The first period occurs during E15 to E17 that is the main onset of neurogenesis, neural migration, and initial axon growth. In this period, at least part of retinal apoptosis is regulated by the low-affinity neurotrophin receptor p75, and most dying cells are observed in the neuroepithelium of the central retina, close to the optic nerve exit.⁴² We could detect ASK1 mRNA after P0, but not at E15 and E17. Thus, it seems to be natural that early PCD was normal in ASK1 KO mice. The second period coincides with the phase of tectal and thalamic innervation and synapse formation.⁴³ In the rat, ~50% of the total RGC population dies in the first postnatal week soon after reaching their target axons, the SC, and the lateral geniculate nucleus.⁴⁴ However, loss of ASK1 did not change the extent of RGC death in this period (Figures 2–4). Since ASK1 is a stress-responsive protein, ASK1-JNK/p38 pathway may not be active in the developing retina. In contrast, Bax, a member of the *BclII* family of cell death regulators, is involved in the control of developmental cell death, but not of photoreceptor degeneration.⁴⁵ There is also another possibility that RGCs are regulated by multiple molecules in addition to ASK1, and single loss of ASK1 has no effect on RGC development. For example, previous studies have shown that brain-derived neurotrophic factor (BDNF) or neurotrophin-4/5 (NT-4/5) injected into the SC promotes the survival of neonatal RGCs.^{46,47} However, BDNF KO and NT-4/5 KO mice have normal RGC numbers.^{21,48} On the other hand, BDNF and NT-4/5 double KO mice showed delayed RGC development.²¹ Thus, there may be the functional redundancy between ASK1 and other molecules, as well as BDNF and NT-4/5, during development. Taken together, multiple survival and death signals seem to be differentially involved in RGC apoptosis according to the developmental stage and pathological conditions. Further studies determining the detailed mechanisms of RGC number control may provide important information for the progress of therapeutic methods in RGC protection and regeneration.

Ischemic retinal injury is now implicated in a number of pathological states, such as retinal artery occlusion, glaucoma, and diabetic retinopathy.^{1–4} Although further studies are needed to identify the precise effect of ASK1 inhibition *in vivo*, our findings raise intriguing possibilities for the management of neural degeneration, as well as infectious diseases and inflammation,⁴⁹ by modifying the activity of the ASK1-p38 pathway.

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