Execution of ASK1-induced Apoptosis by the Mitochondria-dependent Caspase Activation

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Running Title: Mitochondria- and Caspase-dependent Apoptosis by ASK1
Summary

ASK1 activates JNK and p38 MAP kinases and constitutes a pivotal signaling pathway in cytokine- and stress-induced apoptosis. However, little is known about the mechanism how ASK1 executes apoptosis. Here we investigated the roles of caspases and mitochondria in ASK1-induced apoptosis. We found that zVAD-fmk, a broad-spectrum caspase inhibitor, mostly inhibited ASK1-induced cell death, suggesting that caspases are required for ASK1-induced apoptosis. Overexpression of ASK1ΔN, a constitutively active mutant of ASK1, induced cytochrome c release from mitochondria and activation of Caspase-9 and Caspase-3 but not of Caspase-8-like proteases. Consistently, Caspase-8-dificient (Casp8⁻/⁻) cells were sensitive to ASK1-induced Caspase-3 activation and apoptosis, suggesting that Caspase-8 is dispensable for ASK1-induced apoptosis. Whereas, ASK1 failed to activate Caspase-3 in Caspase-9-dificient (Casp9⁻/⁻) cells. Moreover, mitochondrial cytochrome c release, which was not inhibited by zVAD-fmk, preceded the onset of Caspase-3 activation and cell death induced by ASK1. ASK1 thus appears to execute apoptosis mainly by the mitochondria-dependent caspase activation.
Introduction

Apoptosis is a highly regulated process essential for the development and homeostasis of multicellular organisms (1-4). Genetic studies of apoptosis in the nematode *Caenorhabditis elegans* have identified crucial regulators of apoptosis (5). *ced-3* and *ced-4* are required for the execution of apoptosis (6, 7), whereas *ced-9* protects cells from undergoing apoptosis (8). The structural and functional homolog of Ced-9 in mammals has been identified as Bcl-2, which belongs to a large family of molecules involved in either pro-apoptotic or anti-apoptotic signals (9, 10). The mammalian counterpart of Ced-3 has been identified as Caspase-3, a member of a large family of intracellular proteases that play central roles in apoptotic machinery (11). Ced-4 homolog has been identified and termed apoptosis protease activating factor 1 (Apaf1), which is essential for the activation of Caspase-9, another Ced-3 homolog (12, 13).

Despite the diversity of pro-apoptotic signals, the activation of caspases is consistently implicated in apoptosis (14, 15). Among the caspase family, the effector caspases represented by Caspase-3 are frequently activated in response to various apoptosis-inducers and essential for the execution and completion of apoptosis in many, but not all, cell-types or death stimuli (16). Pathways leading to activation of the effector caspases have been identified to be either dependent or independent on the mitochondrial release of cytochrome c into the cytosol (17). The released cytochrome c, together with dATP, binds to Apaf-1, which in turn activates an initiator caspase, Caspase-9 (13). Another initiator caspase, Caspase-8, mainly mediates cytochrome c-independent death signal, which is initiated upon ligation of death receptors (18, 19). These two pathways converge on the activation of the effector
caspases followed by cleavage of a variety of apoptotic substrates.

Activation of the effector caspases appears to be tightly regulated by a divergent signaling mechanism including the members of Bcl-2 family; e.g., a pro-apoptotic Bcl-2 family member, Bid, is cleaved by Caspase-8 and the truncated Bid induces the mitochondrial cytochrome c release (20, 21). In addition to caspase and Bcl-2 families, a number of signaling molecules have been suggested to regulate apoptosis (22). Members of the mitogen-activated protein (MAP) kinase family are involved in signal transduction of apoptosis as well as cell growth and differentiation (23, 24). Two different MAP kinase cascades that converge on c-Jun N-terminal kinase (JNK; also known as SAPK, stress-activated protein kinase) and p38 MAP kinase are preferentially activated by cytotoxic stressors such as UV radiation, X-ray, heat shock and osmotic shock, and by proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (25-27). The possible roles of JNK pathway in apoptosis signaling have been demonstrated by knockout mouse studies. Mice lacking the JNK3 gene were reported to exhibit marked reduction in excitotoxicity-induced apoptosis of hippocampal neurons (28). JNK2 was shown to be required for apoptosis of immature thymocytes induced by anti-CD3 antibody but not for activation-induced cell death of mature T cells (29). Compound mutant mice lacking the JNK1 and JNK2 genes suggested that JNK1 and JNK2 regulate region-specific apoptosis during early brain development (30).

Apoptosis signal-regulating kinase 1 (ASK1) is a ubiquitously expressed MAPK kinase kinase (MAPKKK) that activates the SEK1-JNK and M KK3/ M KK6-p38 signaling cascades (31). Overexpression of ASK1 in epithelial cells in a low serum condition induced apoptosis, and in ovarian cancer cells expression of a kinase-inactive mutant of ASK1 inhibited microtubule-interfering agent-induced apoptosis,
suggesting that ASK1 plays a role in the mechanism of stress-induced apoptosis (31-33). Although moderate expression of a constitutively active form of ASK1 induced neuronal differentiation in naive PC12 cells (34), overexpression of ASK1 induced apoptosis in nerve growth factor (NGF)-differentiated PC12 cells and primary rat sympathetic neurons (35). Moreover, dominant-negative ASK1 reduced the neuronal apoptosis induced by NGF withdrawal from these cells (35). ASK1 was activated upon treatment with TNF-α or agonistic anti-Fas antibody, and a kinase-inactive mutant of ASK1 reduced TNF-α- and Fas-induced JNK activation and apoptosis, suggesting that ASK1 is a pivotal component in cytokine-induced apoptosis as well (31, 36, 37). However, it is still unknown how ASK1-induced apoptosis is executed particularly in relation to caspase activities.

Here we show that ASK1-induced apoptosis is dependent on caspase activities and that the mitochondria-dependent apoptosis pathway is the main mechanism of execution of ASK1-induced apoptosis.
Experimental Procedures

Cell Culture
Mv1Lu mink lung epithelial cells (CCL-64; American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 units/ml penicillin G in a 5% CO2 atmosphere at 37 °C. Mouse embryonic fibroblasts (MEFs) were obtained from E12.5 embryos and cultured in DMEM containing a higher concentration of glucose (4.5 mg/ml) supplemented with 10% FCS and 100 units/ml penicillin G in a 5% CO2 atmosphere at 37 °C. Caspase-8-deficient (Casp8 \(^{-/-}\)) and wild type (Casp8 \(^{+/+}\)) MEFs\(^2\) (38) and Caspase-9-deficient (Casp9 \(^{-/-}\)) and wild type (Casp9 \(^{+/+}\)) MEFs (39) were obtained from E10.5 and E15.5 embryos, respectively, and cultured under the same condition as described above.

Antibodies and Reagents
A monoclonal antibody to the hemagglutinin (HA) tag (clone 3F10) was purchased from Roche Molecular Biochemicals. Monoclonal antibodies to Caspase-3, cytochrome c, and α-tubulin were purchased from Transduction Laboratories, Pharmingen, and Harlan Sera-Lab, respectively. zVAD-fmk was purchased from Peptide Institute, Inc.

Infection of recombinant adenoviruses
Recombinant adenoviruses encoding HA-tagged ASK1 mutants and β-galactosidase were constructed as described (32). Cells were plated in assay plates
and allowed to grow in DMEM supplemented with 10% FCS for overnight. Mv1Lu cells and MEFs were washed twice with phosphate-buffered saline (PBS) and infected with recombinant adenoviruses in DMEM supplemented with 1% and 0.1% FBS, respectively. Nearly 100% infection of recombinant adenoviruses to Mv1Lu cells and MEFs can be achieved at a multiplicity of infection (m.o.i.) of 100 and 400, respectively, as determined by β-galactosidase staining (data not shown).

**Determination of cell viability**

Cell viability of Mv1Lu cells was determined by trypan blue exclusion assay. Briefly, cells were washed with PBS, trypsinized, and collected by centrifugation. The cell pellets were resuspended in 0.4% trypan blue in PBS and the proportion of dead blue cells was determined. For MEFs, cell viability was determined using a Cell Counting Kit-8 (Dojindo), in which 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) was used as a substrate. Relative number of surviving cells was determined in duplicate by estimating the value of uninfected cells as 100%.

**Western blot analysis**

Cells were lysed in a lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS. Cell extracts were clarified by centrifugation and resolved on SDS-polyacrylamide gel electrophoresis followed by electroblotting onto polyvinylidene difluoride membranes. After blocking with 5% skim milk in TBS-T (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 0.05% Tween 20) for 1 h, the membranes were probed with antibodies. The antibody-antigen complexes were detected using the ECL system (Amersham Pharmacia Biotech).
Annexin V staining

Cells were trypsinized, washed with PBS, and resuspended in a binding buffer containing 10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂. After 15 min of incubation with Annexin V-FITC and propidium iodide (PI; Pharmingen) at room temperature, the fluorescence emitted by cells (10,000 cells/sample) was analyzed using a FACScan flow cytometer (Becton Dickinson).

DNA fragmentation analysis

Approximately 2 X 10⁶ cells were lysed with 200µl of a buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 0.5% Triton X-100. Cell extracts were clarified by centrifugation at 15,000 rpm for 5 min. The lysate was incubated with 0.2 mg/ml of proteinase K at 42°C for 1 h. DNA was purified by standard phenol-chloroform extraction and ethanol precipitation. Dry DNA pellets were then resuspended in TE buffer (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA) containing 0.2 mg/ml ribonuclease A. Samples were loaded on a 2% agarose gel and visualized by ethidium bromide staining.

Measurement of caspase activities

Approximately 4 X 10⁵ cells were used for measurement of caspase activities. Caspase-3-like activity was measured by a CPP32/Caspase-3 Fluorometric Protease Assay Kit (MBL) following the manufacturer’s instructions, in which a fluorogenic synthetic peptide DEVD-7-amino-4-trifluoromethyl coumarin (AFC) was used as a substrate. Caspase-8- and -9-like activities were measured using IETD-AFC and LEHD-AFC (Calbiochem), respectively. The fluorescence of the released
AFC was measured with an excitation wavelength of 360 nm and an emission wavelength of 530 nm.

**Detection of cytochrome c release**

Approximately $1 \times 10^7$ cells were trypsinized and collected by centrifugation. The cell pellets were washed with PBS and resuspended in 125 µl of buffer containing 250 mM sucrose, 20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride. The cells were then homogenized with 30 strokes of a homogenizer (Wheaton). The mitochondria-enriched fraction was pelleted by centrifugation at 15,000 rpm for 15 min. The supernatant was subjected to Western blot analysis with a monoclonal antibody to cytochrome c.
Results

Characterization of ASK1-induced cell death

To investigate the mechanism regulating an execution process of ASK1-induced apoptosis, we first characterized the death-inducing activity of ASK1 in Mv1Lu cells and primary MEF cells. Mv1Lu and MEFs were infected with recombinant adenoviruses encoding a hemagglutinin (HA) epitope-tagged constitutively active mutant of ASK1 (Ad-ASK1ΔN) or an HA-tagged catalytically inactive mutant of ASK1 (Ad-ASK1KM) (32). Expression of ASK1ΔN and ASK1KM was detectable as early as 8 h after infection by Western blot analysis (data not shown) and was clearly detected in an m.o.i.-dependent manner after 24 h in both cells (Fig 1, lower panel and data not shown). Forty-eight h after infection, ASK1-induced cell death became apparent in Mv1Lu as determined by a trypan blue exclusion assay (Fig. 1, upper panel). Only ASK1ΔN but not ASK1KM induced a significant cell death, confirming that the death-inducing effect of ASK1 depends on its enzymatic activity. Although a longer time period was required (3 to 6 days culture after infection), adenovirus-mediated expression of ASK1ΔN but not ASK1KM strongly induced cell death in MEFs as well (data not shown).

To examine whether the ASK1-induced cell death is accompanied by apoptotic features, distribution of phosphatidyl serine (PS) on the plasma membrane and fragmentation of chromosomal DNA were analyzed by Annexin V staining and DNA laddering assay, respectively. Compared with uninfected or Ad-ASK1KM-infected cells, Ad-ASK1ΔN-infected cells displayed an increased Annexin V positive population in Mv1Lu (Fig. 2A). Of note, Annexin V positive but PI negative population
was also increased in the ASK1ΔN-expressing cells, suggesting that ASK1-induced cell death follows an ordered apoptotic process. When the cytoplasmic small fragmented DNA was extracted and analyzed by agarose gel electrophoresis, DNA fragmentation was observed in Ad-ASK1ΔN-infected MEFs, but not in uninfected or Ad-ASK1KM-infected cells (Fig. 2B). These findings indicate that the expression of ASK1ΔN effectively induces apoptosis in Mv1Lu cells and MEFs, and that these cells provide useful models to investigate the mechanism of ASK1-induced apoptosis.

**Caspase activities in ASK1-induced apoptosis**

Since members of the caspase family are crucial mediators of apoptosis (14-16), we examined the requirement of caspase activities for ASK1-induced apoptosis using zVAD-fmk, a broad-spectrum caspase inhibitor. We confirmed that addition of zVAD-fmk did not reduce the adenovirus-mediated expression of ASK1ΔN in Mv1Lu cells (Fig. 3, lower panel). When cell death was determined by trypan blue exclusion assay, ASK1-induced death was significantly inhibited in the presence of zVAD-fmk, suggesting that caspase activity is required for ASK1-induced apoptosis (Fig. 3, upper panel). Consistently, proteolytic processing of pro-Caspase-3 was induced by the expression of ASK1ΔN but not ASK1KM in an m.o.i.-dependent manner, suggesting that Caspase-3 is activated during the course of ASK1-induced apoptosis (Fig. 4).

To investigate the involvement of initiator caspases in ASK1-induced apoptosis, we next examined the activities of Caspase-8- and -9-like proteases in comparison with Caspase-3-like activity induced by the expression of ASK1ΔN.
Fluorogenic synthetic peptides, DEVD-AFC, IETD-AFC, and LEHD-AFC were utilized as substrates to assess the activities of Caspase-3-, Caspase-8-, and Caspase-9-like proteases, respectively. Consistent with the ASK1ΔN-induced processing of pro-Caspase-3 (Fig. 4), expression of ASK1ΔN but not ASK1KM strongly induced Caspase-3-like activity 48 h after the infection in Mv1Lu cells (Fig. 5A, left panel). At this time point, a substantial level of Caspase-9-like activity was also induced by the expression of ASK1ΔN, whereas only a marginal activation of Caspase-8-like proteases was detected (Fig. 5A, middle and right panels). To exclude a possibility that activation of Caspase-8-like proteases occurred earlier, we examined the time course of activities of Caspase-8- and Caspase-3-like proteases (Fig. 5B). Activation of Caspase-3-like proteases was first detected 28 h after the infection of ASK1ΔN in Mv1Lu cells. In contrast, no significant activation of Caspase-8-like proteases was detected throughout the course of ASK1-induced apoptosis, suggesting that activation of Caspase-8-like proteases is not involved in ASK1-induced apoptosis.

**Requirement of Caspase-9 but not Caspase-8 for ASK1-induced Caspase-3 activation and apoptosis**

We next assessed the requirement of Caspase-8 and -9 for ASK1-induced Caspase-3 activation and apoptosis using MEFs deficient for Caspase-8 (Casp8<sup>-/-</sup>) or -9 (Casp9<sup>-/-</sup>) (39). Expression of ASK1ΔN clearly induced Caspase-3-like activity in Casp8<sup>-/-</sup> MEFs as well as in wild type (Casp8<sup>+/+</sup>) MEFs (Fig. 6A). Consistently, Casp8<sup>-/-</sup> and Casp8<sup>+/+</sup> MEFs were equally sensitive to ASK1-induced cell death as
determined by a modified 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Fig. 6B). Together with no obvious increase in Caspase-8-like activity in ASK1ΔN-expressing Mv1Lu cells (Fig. 5), these results strongly suggest that Caspase-8 is dispensable for ASK1-induced activation of Caspase-3 and apoptosis.

In contrast, ASK1-dependent activation of Caspase-3-like proteases was completely absent in Casp9 −/− MEFs (Fig. 7A), suggesting that Caspase-9 is prerequisite to ASK1-induced activation of Caspase-3. On the other hand, Casp9 −/− MEFs were still sensitive to ASK1-induced cell death, although less sensitive than the wild type (Casp9 +/+ ) MEFs (Fig. 7B). These results suggest that other caspases, which lack DEVDase activity, may be involved in the execution of apoptosis induced by ASK1 (see Discussion).

Involvement of cytochrome c release in ASK1-induced apoptosis

Since activation of Caspase-9 requires the mitochondrial release of cytochrome c into the cytosol (13), we examined whether cytochrome c release occurs in ASK1-induced apoptosis. We isolated the cytosolic fractions from ASK1ΔN- or ASK1KM-expressing cells and detected the released cytochrome c from mitochondria by Western blot analysis. The expression of ASK1ΔN but not ASK1KM resulted in release of cytochrome c from mitochondria in Mv1Lu cells and MEFs (Fig. 8A and data not shown), clearly demonstrating the involvement of cytochrome c release in ASK1-induced apoptosis. Importantly, addition of zVAD-fmk did not inhibit the cytochrome c release from mitochondria, suggesting that the cytochrome c release is a trigger, rather than a consequence, of the caspase activation in ASK1-induced
apoptosis. Consistent with this notion, ASK1ΔN-induced cytochrome c release was detected as early as 18 h after the infection of Ad-ASK1ΔN and increased thereafter (Fig. 8B). Since no apparent cell death was detected within 30 h in ASK1ΔN-expressing Mv1Lu cells (data not shown), cytochrome c release obviously preceded the onset of cell death and was likely to be one of the determinants of ASK1-induced apoptosis. Taken together, ASK1 appears to induce apoptosis mainly via mitochondrial pathway followed by Caspase-9 and -3 activation.
Discussion

In the present study, we investigated the roles of mitochondria and caspases in the execution of ASK1-induced apoptosis. We found that caspase activities are required for ASK1-induced apoptosis at least in two different cell-types, epithelial cells (Mv1Lu) and fibroblasts (MEFs). In the presence of a broad-spectrum caspase inhibitor, zVAD-fmk, ASK1-induced apoptosis was almost suppressed (Fig. 3), suggesting that ASK1-induced apoptosis is largely dependent on caspase activities. Based on the requirement of caspase activities, we first presumed that ASK1 might kill cells through death receptor-induced direct activation of Caspase-8, since activation of JNK has been reported to lead to de novo synthesis of pro-apoptotic proteins, e.g. Fas ligand (FasL), via c-Jun-dependent transcription (40, 41). In fact, expression of an activated form of MEKK1, another MAPKKK in the JNK pathway, results in apoptosis of Jurkat T cells and PC12 cells in a FasL induction-dependent manner (40, 41). However, ASK1 induced only a limited increase in the Caspase-8-like activity (Fig. 5), and MEFs deficient in Caspase-8 were still sensitive to ASK1-induced Caspase-3 activation and apoptosis (Fig. 6). These results clearly demonstrate that Caspase-8 is not a minimum requirement for ASK1-induced apoptosis at least in these cells.

In contrast, another initiator caspase, Caspase-9, appears to be crucial for Caspase-3 activation in ASK1 signaling. Caspase-9 was activated by the expression of ASK1ΔN (Fig. 5), and more convincingly, ASK1-induced Caspase-3 activation was completely absent in Casp9<sup>−/−</sup> MEFs (Fig. 7A). These results strongly suggest that Caspase-9 is indispensable for ASK1-induced activation of Caspase-3 and exclude a possibility that Caspase-3 is directly activated downstream of the
ASK1-JNK and/or -p38 axes. Unexpectedly, however, Casp9 \(^{-/-}\) MEFs were not completely resistant to ASK1-induced apoptosis (Fig. 7B). An explanation for the discrepancy between the complete absence of ASK1-induced Caspase-3-like activity and the limited resistance to apoptosis induced by ASK1 in Casp9 \(^{-/-}\) MEFs is that other caspases, which lack DEVDase activity, may participate in the execution of ASK1-induced apoptosis or may be activated in compensation for Caspase-9 deficiency (42). A candidate for such among effector caspases is Caspase-6, since the optimal tetrapeptide sequence for Caspase-6 is VEHD and considerably differs from DEVD, a preferential sequence for Caspase-3 and –7 (43). Alternatively, incomplete inhibition of ASK1-induced apoptosis by zVAD-fmk (Fig. 3) may suggest the possible involvement of caspase-independent mechanism in ASK1-induced apoptosis, including a recently identified caspase-independent effector of apoptosis, AIF (apoptosis inducing factor) (44). On the other hand, Casp9 \(^{-/-}\) MEFs were in fact less sensitive to ASK1-induced apoptosis than Casp9 \(^{+/+}\) MEFs (Fig. 7B). The observed difference in the sensitivity to ASK1-induced apoptosis is likely to be attributable to the lack of the increase in DEVDase activity in Casp9 \(^{-/-}\) MEFs.

The mitochondrial release of cytochrome c into the cytosol is required for activation of capase-9 (13). Consistently, we observed cytochrome c release in ASK1-induced apoptosis (Fig. 8). Of note, addition of zVAD-fmk, which effectively inhibited the cell death induced by ASK1 (Fig. 3), caused no significant reduction in cytochrome c release, indicating that caspase activities are not required for ASK1-induced release of cytochrome c. Moreover, ASK1-induced release of cytochrome c obviously preceded the onset of cell death. These results strongly suggest that cytochrome c release is not a consequence but a trigger of ASK1-induced caspase
activation. Mitochondrion may thus be a direct target of death signals mediated via ASK1-JNK and/or -p38 axes.

The molecular target for the activated JNK or p38 to induce cytochrome c release from mitochondria remains to be elucidated. Candidates as such are the Bcl-2 family members, which play pivotal roles in decision of cell death and survival mainly at the level of mitochondria (9, 10). Recently, Bcl-2 was reported to be phosphorylated in a normal physiologic process at G2/M as well as in response to microtubule-interfering agents via the ASK1-JNK1 pathway (45). The phosphorylated Bcl-2 is suggested to lose its anti-apoptotic activity, and therefore cells appear to be susceptible to death signals (45). It will be interesting to study whether other members in the Bcl-2 family may also be regulated by ASK1-JNK and/or -p38 signals.

In conclusion, the results presented here strongly suggest that the mitochondria-initiated activation of effector caspases is a main mechanism operating in the execution of ASK1-induced apoptosis. However, further investigations will be needed to elucidate the issue of cell-type specificity in ASK1 signaling and the possible involvement of other signaling pathways mediating ASK1-induced death signals.
Acknowledgments

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References

19. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O’Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter,


Footnotes

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1. Abbreviations used in this paper: Apaf1, apoptosis protease activating factor 1; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; TNF, tumor necrosis factor; ASK1, apoptosis signal-regulating kinase 1; MAPKKK, mitogen-activated protein kinase kinase kinase; NGF, nerve growth factor; SCG, superior cervical ganglia; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; MEF, mouse embryonic fibroblast; Casp, Caspase; HA, hemagglutinin; AFC, 7-amino-4-trifluoromethyl coumarin; Ad, adenovirus; PS, phosphatidyl serine; AIF, apoptosis inducing factor.

2. Sakamaki K. and Yonehara S., Manuscript in preparation
Figure Legends

Fig. 1. Constitutively active ASK1 (ASK1ΔN)-induced death of Mv1Lu cells. Upper panel, Mv1Lu cells were left uninfected or infected with the indicated m.o.i. of a recombinant adenovirus encoding HA-tagged ASK1ΔN (Ad-ASK1ΔN) or HA-tagged ASK1KM (Ad-ASK1KM). Forty-eight h after the infection, cell death was detected by trypan blue exclusion assay. Results are the means of three independent experiments ± S. E. Lower panel, cells were lysed 24 h after the infection and immunoblotted with an anti-HA antibody to detect the adenovirus-mediated expression of ASK1ΔN and ASK1KM.

Fig. 2. Apoptotic features of ASK1ΔN-induced cell death. A, ASK1ΔN-induced increase in the Annexin V positive population in Mv1Lu cells. Mv1Lu cells were left uninfected or infected at m.o.i. of 100 with Ad-ASK1ΔN or Ad-ASK1KM. Forty-eight h after the infection, cells were stained with Annexin V-FITC and PI and subjected to flow cytometry analysis. Fluorescence dot blots of Annexin V positive (horizontal axis) and PI positive (vertical axis) cells are shown. B, ASK1ΔN-induced DNA fragmentation in MEFs. MEFs were left uninfected (-) or infected with Ad-ASK1ΔN or Ad-ASK1KM at m.o.i. of 400. Four days after the infection, cytoplasmic DNA was isolated and analyzed by 2% agarose gel electrophoresis. As a positive control, cells were exposed to 3 μg/ml of anisomysin for 17 h (indicated as Aniso).

Fig. 3. Effect of zVAD-fmk on ASK1ΔN-induced apoptosis. Mv1Lu cells
were infected with Ad-ASK1ΔN or Ad-β-galactosidase (Ad-β-gal) at m.o.i. of 100 in the presence (+) or absence (-: containing the equivalent volume of Me₂SO) of 50 μM zVAD-fmk. Cells were lysed 24 h after the infection and immunoblotted with an anti-HA antibody to detect the expressed ASK1ΔN (Lower panel). Forty-eight h after the infection, cell death was detected by trypan blue exclusion assay. Results are the means of three independent experiments ± S. E. (Upper panel).

**Fig. 4.** Processing of pro-Caspase-3 induced by the expression of ASK1ΔN. MEFs were left uninfected or infected with the indicated m.o.i. of Ad-ASK1ΔN or Ad-ASK1KM. Four days after the infection, cells were lysed and immunoblotted with a monoclonal antibody to Caspase-3. As a positive control for processing of pro-Caspase-3, cells were exposed to 5 μg/ml of anisomycin for 12 h (indicated as Aniso).

**Fig. 5.** Caspase activities induced by the expression of ASK1ΔN. A, activation of Caspase-3-, -8-, and -9-like proteases in Mv1Lu cells. Mv1Lu cells were left uninfected (-) or infected at m.o.i. of 100 with Ad-ASK1ΔN (ΔN) or Ad-ASK1KM (KM). Forty-eight h after the infection, cells were lysed and the cytosolic extracts were used to measure the activities of Caspase-3-, -8-, and -9-like proteases using DEVD-AFC, IETD-AFC, and LEHD-AFC as substrates, respectively. The cleavage activity is shown as fold increase relative to that of an extract from the uninfected cells. Results are the means of duplicate determinations ± S. E. from a representative experiment. B, time course activation of Caspase-3- and -8-like proteases in Mv1Lu cells. Mv1Lu cells were infected with Ad-ASK1ΔN (m.o.i. 100) at
0 h. At different time points after the infection, the activities of Caspase-3- and -8-like proteases were measured using DEVD-AFC and IETD-AFC as substrates, respectively. The cleavage activity is shown as fold increase relative to that of an extract from the uninfected cells. Results are the means of duplicate determinations ± S. E. from a representative experiment.

Fig. 6. Caspase-3-like activity and apoptosis induced by the expression of ASK1ΔN in Casp8^{−/+} MEFs. A, Caspase-3-like activity in Casp8^{−/−} MEFs. Wild type (Casp8^{+/+}) and Casp8^{−/−} MEFs were left uninfected (-) or infected at m.o.i. of 400 with Ad-ASK1ΔN (ΔN) or Ad-ASK1KM (KM). Four days after the infection, cells were lysed and the cytosolic extracts were used to measure the activity of Caspase-3-like proteases using DEVD-AFC as a substrate. The cleavage activity is shown as fold increase relative to that of an extract from the uninfected cells. Results are the means of duplicate determinations ± S. E. from a representative experiment. B, cell viability of Casp8^{−/−} MEFs. Casp8^{+/+} and Casp8^{−/−} MEFs were left uninfected (-) or infected with Ad-ASKΔN (ΔN) or Ad-ASK1KM (KM) at m.o.i. of 400. Six days after the infection, the relative number of surviving cells was determined using a Cell Counting Kit-8. Results are the means of duplicate determinations ± S. E. from a representative experiment.

Fig. 7. Caspase-3-like activity and apoptosis induced by the expression of ASK1ΔN in Casp9^{−/−} MEFs. A, Caspase-3-like activity in Casp9^{−/−} MEFs. Wild type (Casp9^{+/+}) and Casp9^{−/−} MEFs were left uninfected (-) or infected with Ad-ASK1ΔN (ΔN) or Ad-ASK1KM (KM) at m.o.i. of 400. Four days after the infection,
cells were lysed and the cytosolic extracts were used to measure the activity of Caspase-3-like proteases using DEVD-AFC as a substrate. The cleavage activity is shown as fold increase relative to that of an extract from the uninfected cells. Results are the means of duplicate determinations ± S. E. from a representative experiment.

B, cell viability of Casp9⁻/⁻ MEFs. Casp9⁺/+ and Casp9⁻/⁻ MEFs were left uninfected (-) or infected with Ad-ASK1ΔN (ΔN) or Ad-ASK1KM (KM) at m.o.i. of 400. Four days after the infection, the relative number of surviving cells was determined using a Cell Counting Kit-8. Results are the means of duplicate determinations ± S. E. from a representative experiment.

**Fig. 8. Cytochrome c release in ASK1ΔN-expressing cells.** A, effect of zVAD-fmk on cytochrome c release in ASK1ΔN-expressing cells. Mv1Lu cells were left uninfected or infected with Ad-ASK1ΔN or Ad-ASK1KM at m.o.i. of 100 in the presence (+) or absence (-: containing the equivalent volume of Me₂SO) of 50 µM zVAD-fmk. Cells were collected 30 h after the infection, and cytosolic fractions separated from mitochondria-enriched fractions were subjected to Western blotting analysis with a monoclonal antibody to cytochrome c (*upper panel*). The blot was reprobed with an anti-α-tubulin antibody to confirm the equal loading of the extracts (*lower panel*). B, time course release of cytochrome c in Mv1Lu cells. Mv1Lu cells were infected with Ad-ASK1ΔN (m.o.i. 100) at 0 h. Cells were collected at the indicated time points after the infection, and cytosolic fractions were subjected to Western blot analysis with a monoclonal antibody to cytochrome c (*upper panel*). The blot was reprobed with an anti-α-tubulin antibody to evaluate the loading of the
extracts (lower panel).
Fig. 1

The figure shows a bar graph illustrating the death percentage (%) of cells infected with different doses of Ad-ASK1ΔN and Ad-ASK1KM at various m.o.i. (multiplicities of infection) levels. The x-axis represents the m.o.i. and the y-axis represents the death percentage.

- m.o.i. 0 (uninfected) shows a very low death percentage.
- Ad-ASK1ΔN at 100 m.o.i. shows a significant increase in death percentage.
- Ad-ASK1ΔN at 200 m.o.i. shows an even higher death percentage.
- Ad-ASK1ΔN at 400 m.o.i. shows the highest death percentage among the infected groups.

The inset image shows a Western blot analysis with bands for HA-ASK1KM and HA-ASK1ΔN, indicating the expression levels of these proteins under different conditions.
Fig. 3
Fig. 4

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<th>m.o.i.</th>
<th>0</th>
<th>100</th>
<th>200</th>
<th>400</th>
<th>400</th>
<th>Aniso</th>
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<tr>
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<td>ΔN</td>
<td>KM</td>
<td>-</td>
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- pro-Caspase-3: 29 kDa
- processed Caspase-3: 18 kDa
Fig. 5

A

![Graph showing relative cleavage activity for DEVD-AFC, IETD-AFC, and LEHD-AFC with treatments ΔN, KM, and - over time (h).](image)

B

![Graph showing relative cleavage activity of IETD-AFC and DEVD-AFC over time (h).](image)
Fig. 6

A

B
Fig. 7

A

B

relative DEVDase activity

surviving cells (%)

- ΔN KM

Casp9 +/-

Casp9 -/-

- ΔN KM

Casp9 +/-

Casp9 -/-
Fig. 8

A

<table>
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<tr>
<th>Infection</th>
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-  cytochrome c ▶
- α-tubulin ▶

B

<table>
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<td>0 18 30 42 h</td>
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-  cytochrome c ▶
- α-tubulin ▶
Execution of ASK1-induced apoptosis by the mitochondria-dependent caspase activation

Takiko Hatai, Atsushi Matsuzawa, Seiji Inoshita, Yoshiyuki Mochida, Takayuki Kuroda, Kazuhiro Sakamaki, Keisuke Kuida, Shin Yonehara, Hidenori Ichijo and Kohsuke Takeda

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