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Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Aβ-induced cell death

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Recent studies have suggested that neuronal death in Alzheimer's disease or ischemia could arise from dysfunction of the endoplasmic reticulum (ER). Although caspase-12 has been implicated in ER stress-induced apoptosis and amyloid-β (Aβ)–induced apoptosis in rodents, it is controversial whether similar mechanisms operate in humans. We found that human caspase-4, a member of caspase-1 subfamily that includes caspase-12, is localized to the ER membrane, and is cleaved when cells are treated with ER stress-inducing reagents, but not with other apoptotic reagents. Cleavage of caspase-4 is not affected by overexpression of Bcl-2, which prevents signal transduction on the mitochondria, suggesting that caspase-4 is primarily activated in ER stress-induced apoptosis. Furthermore, a reduction of caspase-4 expression by small interfering RNA decreases ER stress-induced apoptosis in some cell lines, but not other ER stress-independent apoptosis. Caspase-4 is also cleaved by administration of Aβ, and Aβ-induced apoptosis is reduced by small interfering RNAs to caspase-4. Thus, caspase-4 can function as an ER stress-specific caspase in humans, and may be involved in pathogenesis of Alzheimer's disease.

Introduction

Recently, it has been reported that some human diseases, such as Alzheimer's disease (AD), Parkinson's diseases, and cystic fibrosis, and neuronal damage by ischemia are related to stress acting on the ER, which leads to intraluminal accumulation of unfolded proteins (Katayama et al., 1999; Wigley et al., 1999; Imai et al., 2000, 2001; Nakagawa et al., 2000; Sato et al., 2001; Tamatani et al., 2001). Stress on the ER can be induced in vitro by depletion of calcium from the ER lumen, inhibition of asparagine N-linked glycosylation, reduction of disulfide bonds, expression of mutant proteins, and ischemia (Imaizumi et al., 2001). ER stress induces three major cellular responses: unfolded protein response (UPR), ER-associated degradation, and apoptosis. Cells exposed to ER stress can up-regulate genes encoding chaperones that facilitate the protein folding process in the ER and reduce overall translation (UPR; Harding et al., 1999; Kaufman, 2002; Forman et al., 2003), or enhance proteasomal degradation of misfolded ER protein in cytosol (Bonifacino and Weissman, 1998; Travers et al., 2000), to reduce the accumulation and aggregation of misfolded proteins, and relieve cells from the stress (Kozutsumi et al., 1988). On the other hand, excessive or long-termed ER stress results in apoptotic cell death, involving nuclear fragmentation,

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Key words: apoptosis; ER stress; caspase-4; Alzheimer's disease; amyloid-β

Abbreviations used in this paper: Aβ, amyloid-β; AD, Alzheimer's disease; ICE, interleukin-1β converting enzyme; RNAi, RNA interference; siRNA, small interfering RNA; TRAF2, tumor necrosis factor receptor–associated factor 2; UPR, unfolded protein response.

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condensation of chromatin, and shrinkage of the cell body (Imaizumi et al., 2001). Several mechanisms that activate apoptotic signaling pathways have been reported. For example, the UPR increases the transcription of CHOP/GADD153 (Brewer et al., 1997), which is closely associated with cell death (Zinszner et al., 1998), recruitment of tumor necrosis factor receptor–associated factor 2 (TRAF2) to activated IRE1α induces c-Jun NH_2-terminal kinase activation (Urano et al., 2000), or calpain activates downstream caspase cascade (Nakagawa and Yuan, 2000). However, little is known about the precise mechanisms to lead to ER stress-induced cell death in humans.

Activation of caspases, a family of cysteine proteases that cleave substrates at specific aspartate residues, is a central mechanism in the apoptotic cell death process (Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998). Most of apoptosis-inducing stimuli lead to release of cytochrome *c* from mitochondria, which binds to Apaf-1 to activate caspase-9 (Li et al., 1997; Zou et al., 1997), one of initiator caspases with a long pro-domain, and then the activated caspase-9 cleaves effector caspases (Li et al., 1997), including caspases 3 and 7 with a relatively short pro-domain, to activate them. Antiapoptotic Bcl-2 family proteins can rescue cells from apoptosis by protecting mitochondria to prevent cytochrome *c* release (Kluck et al., 1997; Yang et al., 1997). Several initiator caspases are known to be activated upstream of the mitochondrial dysfunction by specific apoptotic stimuli. For example, Fas stimulation can activate caspase-8 (Fernandes-Alnemri et al., 1996; Muzio et al., 1996), which cannot be inhibited by Bcl-2 (Scaffidi et al., 1998). Among 14 known caspases, caspase-12 seems to be involved in signaling pathways specific to ER stress-induced apoptosis (Nakagawa et al., 2000). Pro–caspase-12 is predominantly localized to the ER, and is specifically cleaved by ER stress. Furthermore, caspase-12–deficient mice show a reduced sensitivity to amyloid-β (Aβ), which is found in brains from Alzheimer's patients (Selkoe, 1986) and shown to cause neuronal cytotoxicity (Yankner et al., 1989). Based on these findings, caspase-12 has been suggested to play an important role in the pathogenesis of AD and to represent a potential target of treatment. However, caspase-12 has only been cloned in the mouse and rat so far, and therefore it is controversial whether similar mechanisms operate in humans (Katayama et al., 1999; Rao et al., 2001; Fischer et al., 2002).

Human genome sequence that is highly homologous to mouse caspase-12 has been identified at the locus within the caspase-1/interleukin-1β converting enzyme (ICE) genes cluster on chromosome 11q22.3 (Fischer et al., 2002), but the gene is interrupted by frame shift and premature stop codon, and also has amino acid substitution in the critical site for caspase activity (Fischer et al., 2002). Therefore, human caspase-12 seems to be lost, and the caspases that substitute for caspase-12 to be activated specifically by ER stress have not been identified in humans so far. We described here that human caspase-4 located within the caspase-1/ICE genes cluster shows similar characteristics to mouse caspase-12. The role of the caspase-4 in ER stress-induced apoptosis and Aβ-induced cell death will be discussed.

Results

Identification of caspase-4 as a gene homologous to caspase-12

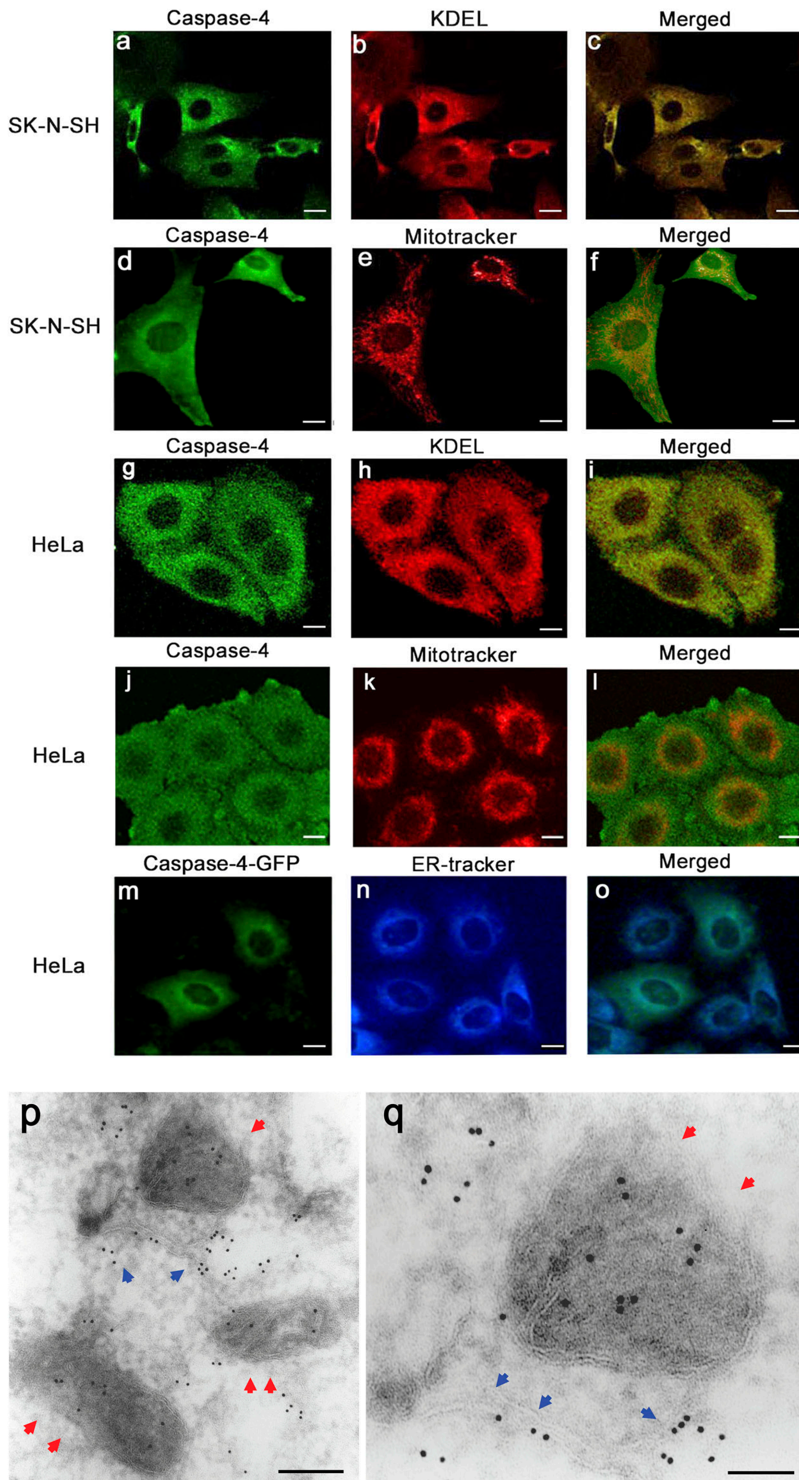
To detect a caspase that was specifically involved in ER stress, we screened human colon cDNA libraries by the plaque hybridization method using the mouse caspase-12 gene as a probe. Human caspase-4 was cloned as the most homologous gene to mouse caspase-12, in agreement with the fact that both molecules belong to the caspase-1/ICE subfamily within the caspase family (Kamens et al., 1995; Lin et al., 2000). Although caspase-5, which has slightly less homology to caspase-12 (caspase-4: 48%; caspase-5: 45%), was also isolated, the screening process yielded much more caspase-4 clones than caspase-5. Because caspase-4 but not caspase-5 was expressed in the cell lines used in this work, which underwent apoptosis in response to ER stress, we assumed that human caspase-4 might functionally substitute for mouse caspase-12 in the human system, and further analyzed the possible role of caspase-4 as a mediator of ER stress-induced apoptosis.

Subcellular localization of caspase-4

First, we studied the subcellular localization of endogenous caspase-4 in SK-N-SH human neuroblastoma cells. Immunofluorescence microscopy showed that immunostaining pattern of caspase-4 strictly overlapped with that of ER markers such as GRP78 and GRP94 (Fig. 1, a–c). Immunoreactivity of caspase-4 was found to overlap only in part with fluorescence signals from Mitotracker (Fig. 1, d–f). These results suggest that caspase-4 was localized predominantly to the ER, and to the mitochondria in addition. The similar results were obtained using HeLa cells (Fig. 1, g–l). When caspase-4 fused with GFP at its COOH terminus was overexpressed in HeLa cells to see the subcellular localization in live cells, most of the fluorescent signals from caspase-4/GFP fusion protein overlapped with those from ER-tracker (Fig. 1, m–o), confirming predominant localization of caspase-4 to the ER by non-immunological method. The immunoelectron microscopic analysis showed that the immunoreactive signals for caspase-4 were found on the ER and mitochondria (Fig. 1, p–r), but much less signals on the nuclei (Fig. 1 r). We also performed biochemical fractionation analysis. Although we could not eliminate contamination of ER marker proteins in the mitochondria-enriched fraction using SK-N-SH cells, probably because we could not disrupt cells homogeneously as the cell line displays heterogeneity in cellular morphology, microsome-enriched fraction does not seem to contain mitochondria and cytosol (Fig. 1 s). Under these conditions, caspase-4 was recovered in both mitochondria-enriched fraction and microsome-enriched fraction, and in cytosolic fraction to a lesser extent (Fig. 1 s), indicating that caspase-4 was surely in microsome-enriched fraction. From these results, we concluded that caspase-4 was localized to the ER, and to the mitochondria in addition, in both SK-N-SH and HeLa cells.

Specific cleavage of caspase-4 by ER stress and Aβ treatments

To examine whether caspase-4 was specifically cleaved by ER stress, we analyzed the cleavage of pro–caspase-4 in re-



r

	Nuclear	Mitochondria	ER	Cytosol	total
Gold	0	359	392	169	920
%	0	39	43	18	100

s

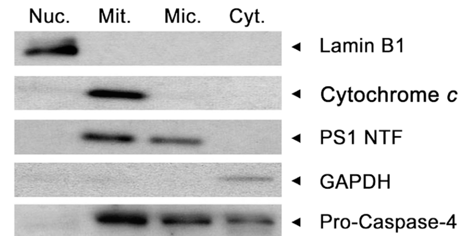


Figure 1. **Localization of caspase-4 in SK-N-SH and HeLa cells.** (a–l) SK-N-SH cells (a–f) or HeLa cells (g–l) were stained with anti–caspase-4 and anti-KDEL antibodies (a and g, caspase-4, green; b and h, KDEL, red; c and i, overlapping, yellow), or with anti–caspase-4 antibody and Mitotracker (d and j, caspase-4, green; e and k, Mitotracker, red; f and l, overlapping, yellow), and observed under a confocal microscope as described in Materials and methods. Anti-KDEL antibody detects both GRP78 and GRP94 (ER markers), whereas Mitotracker stains the mitochondria. (m–o) HeLa cells were transfected with a caspase-4–GFP fusion gene, and were stained with ER-tracker (m, caspase-4, green; n, ER-tracker, blue; o, overlapping, blue-green), and observed under a nonconfocal fluorescence microscope. Bars, 5 μm. (p and q) Immunoelectron microscopic analysis was performed for SK-N-SH cells as described in Materials and methods. Photograph shown in panel q is the enlarged image of a part of photograph p. Gold grains showed the immunoreactivity of caspase-4, and blue and red arrows showed the ER and mitochondria, respectively. Bars: (p) 200 nm; (q) 90 nm. (r) Gold grains observed on indicated organelles in immunoelectron microscopic analysis were counted and displayed. (s) Biochemical fractionation was performed as described in Materials and methods, and analyzed by Western blotting using the indicated antibodies. Lamin B1, nuclear marker; cytochrome *c*, mitochondrial marker; presenilin-1 NH_2-terminal fragment (PS1 NTF), microsomal marker; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cytosolic marker.

sponse to several apoptotic stimuli (Fig. 2 a). We found that cleavage of pro–caspase-4 was induced in SK-N-SH cells by treatment with tunicamycin and thapsigargin, both of which caused ER stress. In contrast, when cells were exposed to non-ER stress inducers such as etoposide, staurosporine, and UV at a dose providing similar extent of cell death to that by tunicamycin and thapsigargin, final cleavage products of pro–caspase-4 (Fig. 2 a, cleaved-caspase-4, arrowhead) was not observed. Although the bands shown by the asterisks in Fig. 2 a, which should be derived from pro–caspase-4 by unknown processing reaction, judging from the data below (Fig. 4 b), were also increased, they were also observed in nontreated cells, so we speculated that the bands were not the final processed form of caspase-4. Under the same conditions, cleavage of caspases 3 and 7, the downstream caspases, was observed regardless of apoptotic stimulations (Fig. 2 a). These results suggest that caspase-4 is specifically activated by apoptotic stimuli inducing ER stress, but not by other stimuli that do not cause ER stress.

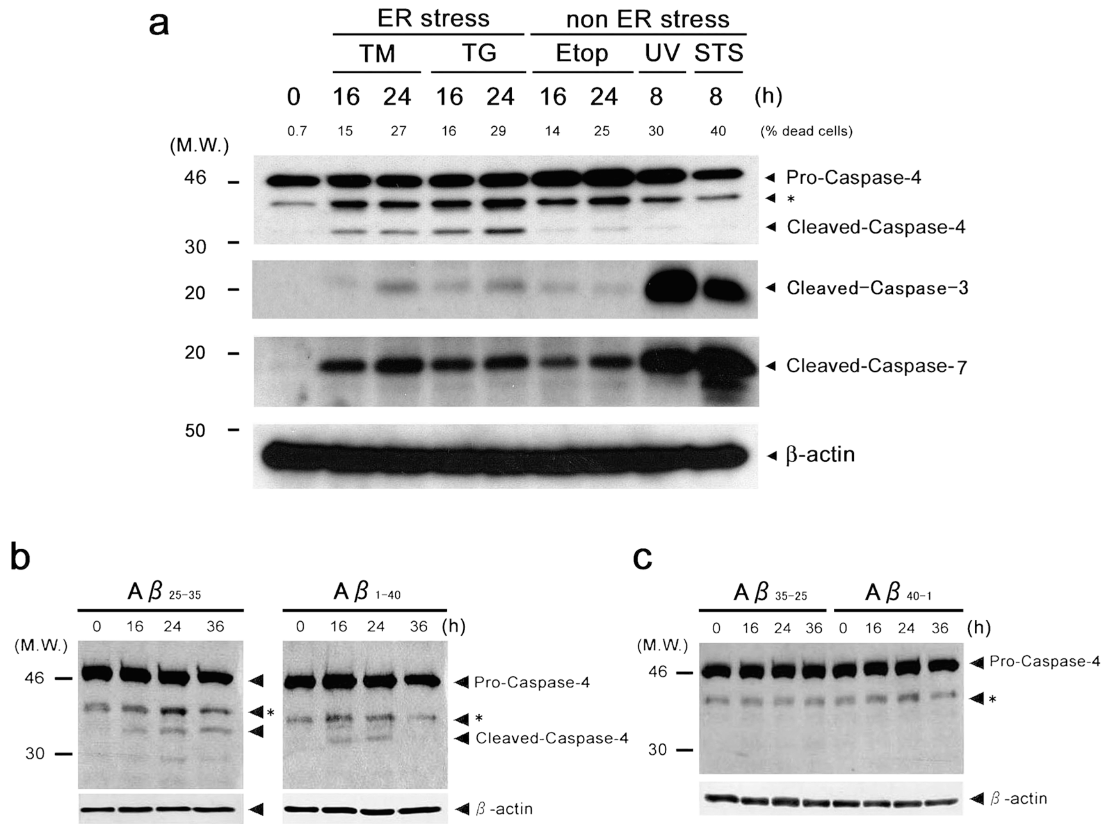


Figure 2. **Specific cleavage of caspase-4 by ER stress and Aβ treatment.** (a) SK-N-SH cells were treated with 1 μg/ml tunicamycin (TM), 0.5 μM thapsigargin (TG), 100 μM etoposide (Etop), or 0.1 μM staurosporine (STS) for indicated periods, or irradiated with 150 J/m^2 UV followed by incubation for indicated periods. Equal amounts of cell lysates (15 μg) were analyzed by Western blotting using anti–caspase-4 antibody (top), anti–caspase-3 antibody (second from top), anti–caspase-7 antibody (third from top), or anti–β-actin antibody (bottom). Positions of pro–caspase-4, cleaved caspase-4, cleaved caspase-3, cleaved caspase-7, and β-actin are indicated. Extent of cell death assessed by MTS assay after incubation for indicated periods are also shown at the top of the gels. (b) SK-N-SH cells were treated with 25 μM synthetic $Aβ_{25-35}$ or 5 μM $Aβ_{1-40}$ peptides for the indicated periods. Equal amounts of cell lysates (15 μg) were analyzed by Western blotting using anti–caspase-4 antibody (top) and anti–β-actin antibody (bottom) as a control. Positions of pro–caspase-4, cleaved caspase-4, and β-actin are indicated. (c) SK-N-SH cells were treated with the reverse peptides (25 μM $Aβ_{35-25}$ and 5 μM $Aβ_{40-1}$, respectively) for the indicated periods, and cleavage of caspase-4 was examined as in panel b. (a–c) Bands marked by asterisks are likely to be derived from pro–caspase-4 by unknown processing reaction.

To address the possibility that caspase-4 contributes to the mechanism of Aβ-induced cell death in humans, we examined the cleavage of caspase-4 in SK-N-SH cells after treatment with Aβ. When cells were incubated with 25 μM $Aβ_{25-35}$ or 5 μM $Aβ_{1-40}$, cleavage of caspase-4 was observed (Fig. 2 b). In contrast, treatment of cells with the reverse peptides ($Aβ_{35-25}$ and $Aβ_{40-1}$, respectively), which were not toxic, did not induce the cleavage of caspase-4 (Fig. 2 c). These results suggest that caspase-4 is activated by neurotoxic Aβ treatment similar to ER stress-induced apoptosis.

Cleavage of caspase-4 in the presence of Bcl-2

To confirm that cleavage of caspase-4 was not due to other caspases activated downstream of the mitochondrial pathway, we examined the effect of overexpression of Bcl-2 and Bcl-x_L on apoptosis induced by tunicamycin. Apoptotic nuclear morphological changes were induced by treatment of vector transfectants of SK-N-SH and of HeLa cells with tunicamycin for 30 h, but such changes were completely suppressed by overexpression of Bcl-2 (Fig. 3) or Bcl-x_L (not depicted), indicating that the apoptotic signaling pathway downstream of mitochondria was not operating in cells with overexpression of these antiapoptotic proteins. However, cleavage of caspase-4 after 16 h of tunicamycin treatment was only slightly affected by overexpression of Bcl-2 (Fig. 3) or Bcl-x_L (not depicted). These results suggested that caspase-4 is largely activated before the activation of effector caspases during ER stress-induced cell death.

Requirement of caspase-4 for ER stress- and Aβ-induced apoptosis

To determine whether caspase-4 is required for ER stress-induced cell death, SK-N-SH cells that expressed endogenous caspase-4 were transfected with small interfering RNA (siRNA) to caspase-4 or GFP as a control. Immunofluorescence analysis showed that the amount of caspase-4 was substantially decreased by incubation for 60 h after transfection with siRNA directed against caspase-4, but immunoreactivity of caspase-4 was not affected by transfection with GFP-siRNA, when compared with nontransfected cells (Fig. 4 a). Western blot analysis also showed that the amount of caspase-4 was decreased by siRNA to caspase-4 (Fig. 4 b). These re-

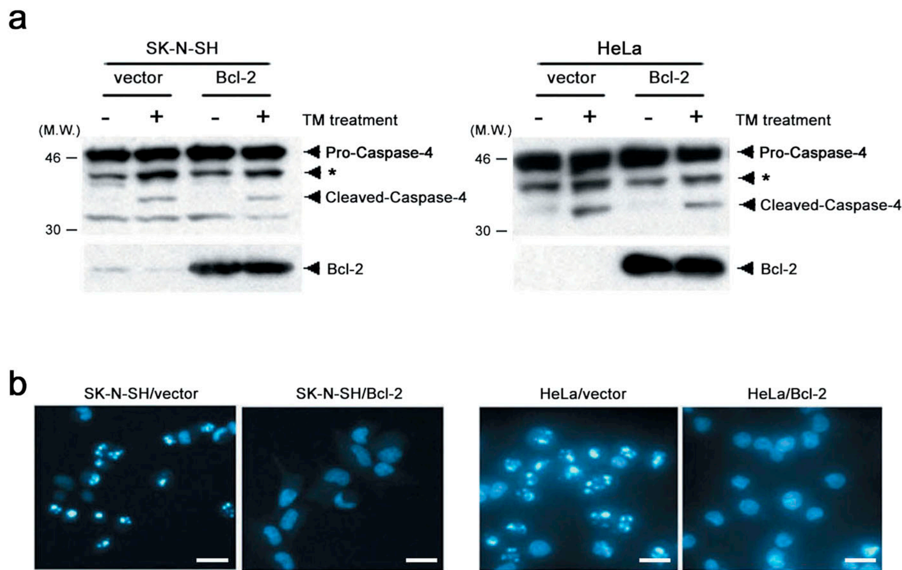


Figure 3. **No effect of Bcl-2 overexpression on ER stress-induced cleavage of caspase-4.** (a) SK-N-SH cells (left) and HeLa cells (right) stably transfected with the vector or a Bcl-2 expression system were incubated with (+) or without (−) 1 μg/ml tunicamycin for 16 h. Equal amounts of cell lysates were analyzed by Western blotting using anti–caspase-4 antibody (top) and anti–Bcl-2 antibody (bottom). Positions of pro–caspase-4, cleaved caspase-4, and Bcl-2 are indicated. Asterisks show processed caspase-4 as described in Fig. 2 a. (b) The indicated cells were treated with 1 μg/ml tunicamycin for 30 h, stained with Hoechst 33342, and observed under a fluorescence microscope. Bars, 25 μm.

sults showed that the siRNA could diminish the amount of caspase-4, and that the antibody used here specifically recognized caspase-4 in immunohistochemical analysis.

We next examined the effect of decrease in caspase-4 level by siRNA on ER stress-induced apoptosis. Assessment of cell death on the basis of morphological changes showed that ~60% of untransfected SK-N-SH cells were killed by treatment with thapsigargin for 40 h. The extent of cell death was unaffected by transfection with siRNA to GFP (Fig. 4 c). In contrast, only ~30% of the cells died after being transfected with caspase-4 siRNA and exposed to the same stimulation with thapsigargin (Fig. 4 c). As shown in Fig. 4 b, treatment with thapsigargin for 24 h yielded lower level of cleaved-caspase-4 in the cells transfected with caspase-4 siRNA than in the cells transfected with GFP-siRNA. Because the amount of cleaved caspase-4 shown in Fig. 4 b seemed to correlate with the extent of cell death in Fig. 4 c, incomplete inhibition of cell death by transfection with caspase-4 siRNA could be due to residual activity of caspase-4. These results indicate that cells with decreased expression of caspase-4 become more resistant to ER stress-induced cell death.

When cell death was examined by the MTS assay, treatment with caspase-4 siRNA, but not with GFP-siRNA, increased the resistance to ER stress-induced cell death (Fig. 4 d). The increase in the resistance to ER stress-induced cell death was also observed when siRNA to caspase-4 with a different sequence (caspase-4 siRNA-b) was used (Fig. 4 d), indicating that the effect was due to the decreased expression of caspase-4, but not by a specific side effect of caspase-4 siRNA that might affect the expression of other genes. On the other hand, the efficiency of cell death induced by etoposide treatment was not significantly affected by both caspase-4 siRNAs (Fig. 4 d). Therefore, caspase-4 is likely to be specifically involved in ER stress-induced cell death.

To know whether caspase-4 is involved in ER stress-induced cell death in other cell lines, we examined the effect of caspase-4 siRNA using HeLa cells. As shown in Fig. 4 e, treatment of HeLa cells with caspase-4 siRNA significantly increased the resistance to ER stress-induced cell death, although the extent of the increase in resistance was less than that observed for SK-N-SH cells. This is probably because some other apoptotic mechanisms might also operate simultaneously in HeLa cells. Therefore, we concluded that caspase-4 is likely to be involved in ER stress-induced cell death at least in part in HeLa cells.

We next examined whether caspase-4 is involved in Aβ-induced cell death. When treated with $A\beta_{25\text{-}35}$, SK-N-SH cells transfected with caspase-4 siRNA showed significant reduction in cell death compared with the cells transfected with GFP-siRNA (Fig. 4 f). From these results presented here, we concluded that caspase-4 is involved in Aβ-induced cell death, as well as in ER stress-induced cell death.

Discussion

It has been known that apoptotic morphological changes are observed in cell death caused by ER stress (Imaizumi et al., 2001). Caspases are activated to transmit apoptotic signals transcending the difference in species (Alnemri et al., 1996). In rodents, caspase-12 mediates apoptosis specifically in response to ER stress (Nakagawa et al., 2000). Although human caspase-12 gene is transcribed into mRNA, mature caspase-12 protein would not be produced, because the gene is interrupted by frame shift and premature stop codon (Fischer et al., 2002). Furthermore, it contains amino acid substitution in the critical site, which leads to loss of function in several caspases (Fischer et al., 2002). Thus, human caspase-12 does not seem to function in ER stress-induced apoptosis, and some other caspases with similar structure might substitute functionally for caspase-12 in humans. The caspase-12 gene is located within a region where caspase-1/ICE subfamily genes cluster (caspases 1, 4, 5, 12 in human and caspases 1, 11, 12 in mouse). No locus with a comparably high homology to rodent caspase-12 could be found in the human genome. Caspases 4 and 5 are located between caspases 1 and 12 in human genome, whereas only caspase-11 is located between caspases 1 and 12 in mouse. Although it is not known why the region in human genome contains gene duplication, caspases 4 and 5 have been thought to function similarly to caspases 11 and 12. Mouse caspase-11

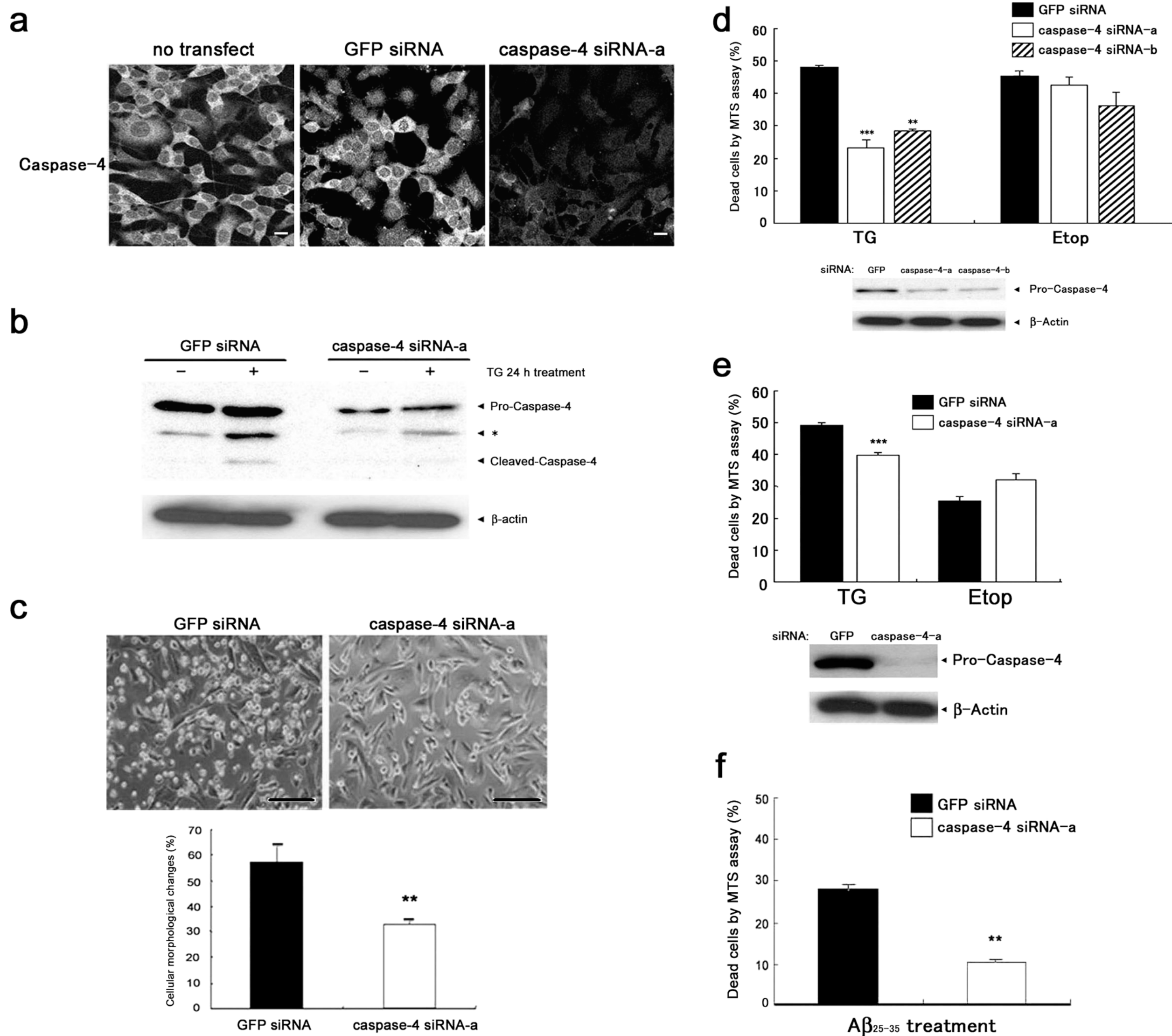


Figure 4. **Decrease in ER stress- or Aβ-induced cell death after siRNA-mediated reduction of caspase-4 expression.** (a) SK-N-SH cells were transfected with siRNA oligos (1 μg oligo/24 well plate) to GFP (control) or caspase-4 (siRNA-a). After incubation for 60 h, cells were fixed and stained with caspase-4 antibodies as described in Materials and methods. Bars, 5 μm. (b) Cells were transfected as in panel a. After incubation for 60 h, cells were incubated with (+) or without (−) 0.5 μM thapsigargin for 24 h. Equal amounts of cell lysates (10 μg) were analyzed by Western blotting using anti–caspase-4 antibody (top) or anti–β-actin antibody (bottom). (c) Top panels show representative phase-contrast images of GFP siRNA-transfected cells (left) and caspase-4 siRNA-a–transfected cells (right) after treatment with 0.5 μM thapsigargin for 40 h. The bottom panel shows the extent of cell death assessed by morphological changes, and expressed as the mean ± SEM for three independent experiments as described in Materials and methods. Asterisks show a significant difference from controls (GFP siRNA-transfected cells): **, indicates $P < 0.01$. Bars, 50 μm. (d) Cells were transfected with the indicated siRNAs, and cell viability after 0.5 μM thapsigargin or 100 μM etoposide treatment for 40 h was estimated by the MTS assay. Results were expressed as the mean ± SEM for three independent experiments. Asterisks show a significant difference from controls: **, indicates $P < 0.01$; ***, indicates $P < 0.001$. Bottom panel shows reduction of caspase-4 level by the indicated siRNAs assessed by Western blotting as described in panel b. (e) HeLa cells were transfected with GFP siRNA or caspase-4 siRNA-a as described in Materials and methods. After incubation for 24 h, cells were incubated with 0.5 μM thapsigargin for 40 h, and then viability was estimated as described in panel d. Each value represents the mean ± SEM for three independent experiments. Asterisks show a significant difference from controls: ***, indicates $P < 0.001$. (f) SK-N-SH cells were transfected with GFP siRNA or caspase-4 siRNA-a. After incubation for 60 h, cells were incubated with 25 μM $A\beta_{25-35}$ peptide for 40 h, and then viability was estimated as described in panel d. Each value represents the mean ± SEM for three independent experiments. Asterisks show a significant difference from controls: **, indicates $P < 0.01$.

is essential for the activation of caspase-1/ICE to promote pro–IL-1β (interleukin-1β) processing (Wang et al., 1996, 1998). On the other hand, caspase-5 is likely involved in processing of pro–IL-1β together with caspase-1/ICE (Martinon et al., 2002) and the caspase-5 gene resembles the mouse caspase-11 in its lipopolysaccharide inducibility (Lin et al., 2000). Therefore, caspase-5 should be the orthologue of caspase-11. Here, the screening process yielded the caspase-4 gene as the homologous gene to mouse caspase-12. Thus, caspase-4 is the best candidate that would function similarly to mouse caspase-12 in ER stress-induced cell death in humans.

Here, we examined the localization of human caspase-4 using several methods. The immunostaining analysis using anti–caspase-4 antibody and fluorescent analysis for caspase-4/GFP fusion protein in Fig. 1 showed the predominant localization of caspase-4 on the ER. On the other hand, the immuno-EM showed the nearly equal distribution of caspase-4 on the ER and mitochondria, and subcellular fractionation showed that caspase-4 was recovered in the microsome-enriched and mitochondria-enriched fractions, and also in cytosolic fraction. Although we could not eliminate contamination of ER marker proteins in the mitochondria-enriched fraction in subcellular fractionation using SK-N-SH cells, microsome-enriched fraction does not seem to contain mitochondria and cytosol. Under these conditions, caspase-4 was recovered in both mitochondria-enriched fraction and microsome-enriched fraction, and amounts of caspase-4 recovered in the microsome-enriched and mitochondria-enriched fractions were comparable to those of ER marker, presenilin-1. Therefore, considering all the results shown in Fig. 1, we concluded that caspase-4 was localized to the ER membrane, and probably to the mitochondria in addition.

Caspase-4 on the ER is supposed to function in ER stress-induced apoptosis similarly to caspase-12. In supporting this hypothesis, caspase-4 was cleaved specifically by ER stress and Aβ-treatment, but not by other apoptotic stimuli including etoposide, staurosporine, and UV. Additionally because Bcl-2 that can completely inhibit the signaling pathway at least downstream from mitochondria, did not prevent the cleavage of caspase-4 by ER stress, it should be most probable that caspase-4 on the ER but not on mitochondria is primarily cleaved. We also showed that reduction of the level of caspase-4 by RNA interference (RNAi) resulted in decrease in ER stress-induced cell death and Aβ-induced cell death, but did not affect cell death induced by etoposide. The characteristics of human caspase-4 shown here are very similar to those of mouse caspase-12 reported previously (Nakagawa et al., 2000), and therefore, caspase-4 is able to substitute the caspase-12 functions in ER stress-induced apoptosis and Aβ-induced cell death. Because caspase-4 was also localized to the mitochondria in addition to the ER membrane, whereas caspase-12 was shown to localize predominantly to the ER, but not to the mitochondria (Nakagawa et al., 2000), caspase-4 might have additional function compared with caspase-12, although the function of caspase-4 on mitochondria is not clear.

Several mechanisms that activate caspase-12 have been proposed in mouse system. For example, calpain, a protease that can be activated by calcium released from ER upon ER stress, starts cleavage of caspase-12 (Nakagawa and Yuan, 2000), caspase-7 activates caspase-12 upon prolonged ER stress (Rao et al., 2001), or TRAF2 mediates caspase-12 activation, which is regulated by IRE1α (Yoneda et al., 2001). It is not clear which mechanism is involved in activation of caspase-4, but because final cleavage products were not observed in cells with activated caspase-7 (Fig. 2 a), activation of caspase-7 does not seem to be enough for full activation of caspase-4. To know the precise mechanism that activate caspase-4, it should be important to find proteins that interact with pro–caspase-4 during ER stress-induced apoptosis.

The inhibition of apoptosis induced by ER stress exposure by RNAi to caspase-4 was incomplete. It is possible that the residual activity of caspase-4 after RNAi would be responsible for the cell death. Alternatively, other apoptotic mechanisms might also operate simultaneously. Several possible pathways have been postulated for ER stress-induced apoptosis. ER stress is reported to activate ASK–c-Jun NH_2-terminal kinase pathway through the IRE1–TRAF2–ASK1 complex formation (Nishitoh et al., 2002). Other signaling pathway is mediated by transcriptional activation of genes encoding proapoptotic function. Activation of stress transducer IRE1, PERK, or ATF6 leads to transcriptional activation of CHOP/GADD153, a bZIP transcription factor that potentiates apoptosis (Oyadomari et al., 2002). Operation of these mechanisms might account for incomplete inhibition of ER stress-induced apoptosis by knockout of caspase-12 and knockdown of caspase-4 in mouse and humans, respectively. It is possible that caspase-dependent mechanism and other mechanisms function in parallel in initiating ER stress-induced apoptosis, and the mechanism that mainly operates could differ depending on cell types. We have shown that the extent to decrease in cell death by decreasing caspase-4 level of SK-N-SH cells was comparable to that reported for caspase-12 knockout mouse (Nakagawa et al., 2000), whereas that of HeLa cells was relatively less. We also find some cells, like HUVEC, in which decrease in caspase-4 did not affect the ER stress-induced apoptosis (unpublished data). Thus, caspase-4 has been shown to function in ER stress-induced apoptosis at least in several cell lines, including SK-N-SH and HeLa cells, but not all cells.

Cell death caused by Aβ treatment was also partially inhibited by RNAi to caspase-4. Although it is controversial whether Aβ-induced cell death involves ER stress-induced apoptosis, the results are consistent that these two types of cell death are mediated by common mechanism at least in part. Recent report described that the cell death induced by Aβ was inhibited by the broad-spectrum caspase inhibitor z-VAD and more specifically by the down-regulation of caspase-2 with antisense oligonucleotides (Haviv et al., 1998). Neuronal culture derived from caspase-2 null mice was also shown to be partially resistant to $A\beta_{1\text{-}42}$ toxicity (Troy et al., 2000). Thus $A\beta_{1\text{-}42}$-induced cell death might be mediated by caspase-2 as well as caspase-12. It is possible that both caspases 2 and 4 are involved in Aβ-induced cell death also in the human system.

Because caspase-4 seems to be responsible for cell death after Aβ treatment, caspase-4 might be involved in pathogenesis of AD. Consistently to this hypothesis, our preliminary analysis showed an increase in cytoplasmic staining for caspase-4 in the pyramidal cell layer of the hippocampal CA1-2 region in AD patients, but not in control brains (unpublished data). All of the AD brains tested had stronger caspase-4 immunoreactivity than disease control brains from patients with other neurodegenerative disorders ($n = 4$ for AD and $n = 3$ for disease control), and the strong staining was remarkable in the pyramidal neurons around deposits of β-amyloid. Increased caspase-4 might elevate the vulnerability of neurons to apoptosis, and therefore may be involved in the pathogenesis of AD.

Bcl-2 family proteins play essential roles in regulating apoptosis. Although antiapoptotic family members (Bcl-2, Bcl-x_L) and multidomain proapoptotic members (Bak, Bax) are thought to function mainly on mitochondria, recent studies suggest that they may also function on the ER where they reside as well. Overexpression of Bcl-2 (Foyouzi-Youssefi et al., 2000; Pinton et al., 2000) or knockout of both Bak and Bax (Scorrano et al., 2003) are reported to reduce ER Ca^{2+} concentration, but other reports showed that Bcl-2 enhanced the retention of Ca^{2+} in the ER lumen (Distelhorst et al., 1996; He et al., 1997). Thus, although it is still controversial, Bcl-2 family members may contribute to regulating ER stress-induced apoptosis on the ER (Ferri and Kroemer, 2001; Scorrano et al., 2003; Zong et al., 2003) in addition to their main function on mitochondria. Our results demonstrate that overexpressed Bcl-2 and Bcl-x_L, which can completely inhibit the signaling pathway at least downstream from mitochondria, did not prevent, but slightly affected, the cleavage of caspase-4 by ER stress. The slight decrease might be due to the absence of its feed back cleavage by downstream effector caspases, such as caspase-3. Therefore, without regard to the mitochondria or the ER where Bcl-2 family functions, activation of caspase-4 should be the primary reaction in ER stress-induced activation of caspases.

In this work, we discovered that caspase-4 plays a key role in ER stress-induced apoptosis in humans. Caspase-4 also seems to act in the β-amyloid–induced cell death, suggesting that human caspase-4 corresponds to rodent caspase-12 to initiate cell death signaling pathway, and that the activation of caspase-4 would mediate neuronal cell death in neurodegenerative disorder. Caspase-4 could be the potential target to develop treatments for such diseases including AD.

Materials and methods

Chemicals and antibodies

We used the following antibodies: anti–caspase-4 mAb (4B9; MBL International Corporation), anti–caspase-4 pAb (Santa Cruz Biotechnology, Inc.), anti-KDEL mAb (10C3; StressGen Biotechnologies), anti–Lamin B1 mAb (L-5; Zymed Laboratories), anti–cytochrome *c* mAb (7H8.2C12; BD Biosciences), anti–glyceraldehyde-3-phosphate dehydrogenase mAb (6G7; Biogenesis), anti–caspase-3 mAb (19; Transduction), anti–caspase-7 mAb (4G2; MBL International Corporation), anti–β-actin mAb (C4; CHEMICON International Inc.), anti–Bcl-2 mAb (#100; BD Biosciences), Alexa 588–conjugated anti–mouse IgG antibody (Molecular Probes), FITC-conjugated anti–goat IgG antibody (Jackson ImmunoResearch Laboratories), gold-conjugated anti–goat IgG antibody (British BioCell), and HRP-conjugated anti–mouse IgG antibody (Cell Signaling). Anti–presenilin-1 polyclonal antibody was raised by immunizing rabbits with a synthetic peptide corresponding to residues 1–14 of human presenilin-1, and was affinity purified using ProtOn Kit1 (MultiplePeptide Systems). The chemical reagents used in this experiment were tunicamycin, thapsigargin, etoposide, staurosporine (Sigma-Aldrich), and ER-tracker and Mitotracker (Molecular Probes). Cytotoxic peptides, $A\beta_{25\text{-}35}$ and $A\beta_{1\text{-}40}$, and their reverse peptides, $A\beta_{35\text{-}25}$ and $A\beta_{40\text{-}1}$, were purchased from Sigma-Aldrich.

Cell culture

Human neuroblastoma SK-N-SH cells and human carcinoma HeLa cells were respectively cultured in α-MEM (Invitrogen) and DME (Invitrogen) both containing 10% FBS, at 37°C under 5% CO_2. For some experiments, these cells were stably transfected with pCAGGS-hBcl-2 (Iwahashi et al., 1997) and pCAGGS-hBcl-x_L (Tagami et al., 2000) to overexpress Bcl-2 and Bcl-x_L, respectively.

cDNA cloning

A human colon cDNA library (Stratagene) was used for hybridization to isolated cDNA homologous to the partial sequence of mouse caspase-12 gene.

Immunofluorescence microscopy

SK-N-SH cells or HeLa cells were incubated with or without Mitotracker probes, and were fixed with 0.1 M phosphate buffer containing 4% PFA for 2 h at 4°C. Cells were incubated with anti–caspase-4 pAb with or without anti-KDEL mAb, followed by FITC- and Alexa 588–conjugated secondary antibodies, respectively. Stained cells were observed under a confocal microscope (model LSM510; Carl Zeiss MicroImaging, Inc.). To determine localization of caspase-4 in live cells, HeLa cells were transfected with caspase-4/GFP fusion gene subcloned into a pcDNA3.1 (Invitrogen) to produce caspase-4 fused with GFP at its COOH terminus, and after 24 h, cells were incubated with ER-tracker probe for 30 min, followed by observation under a fluorescence microscope (model IX71; Olympus).

Immuno-EM

Immuno-EM was performed essentially as described previously (Miyake et al., 2002). In brief, SK-N-SH cells cultured on a 15-cm dish were fixed with 4% PFA in PBS, pH 7.2, at RT for several hours. After harvesting the cells, they were washed with 30 mM Hepes buffer, pH 7.4, several times. The cells were resuspended in 10% gelatin in 30 mM Hepes buffer, pH 7.4, at 37°C. After centrifugation to recover cells, gelatin was solidified on ice. Blocks for ultracryotomy were prepared and infused with 20% polyvinylpyrrolidone/1.84 M sucrose overnight at 4°C. Ultrathin sections were collected on nickel grids and immunostained with anti–caspase-4 pAb. The sections were incubated with gold-conjugated anti–goat IgG antibody (gold particles, 10-nm diam) for 1 h at RT. Grids were contracted in 2% uranyl acetate and examined on a transmission electron microscope (model CM10; Philips).

Subcellular fractionation

SK-N-SH cells cultured on a 15-cm dish were washed twice with PBS, harvested, and suspended in buffer A (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.32 M sucrose, 0.1 mM PMSF) for 5 min on ice. Then the cells were passed through a 25-gauge needle 13 times and centrifuged at 500 *g* for 10 min to collect a crude nuclear pellet. The supernatant was centrifuged at 1,200 *g* for 10 min to yield a mitochondria-enriched pellet, which contained mitochondria and microsome as shown in Fig. 1 s. This supernatant was further centrifuged at 100,000 *g* for 60 min to yield a microsomal pellet and a cytosolic fraction. All of the pellets were dissolved in buffer A containing 1% SDS. Equal volume of each fraction was subjected to Western blotting as described below, using indicated antibodies.

Western blot analysis

Cells treated with the indicated reagents were washed with PBS, harvested, and lysed in TNE buffer (10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 150 mM NaCl, 1 mM PMSF) containing 0.5% NP-40. Equal amounts of protein were subjected to 12% SDS-PAGE and transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% BSA and was incubated with each primary antibody, followed by incubation with an HRP-conjugated secondary antibody. Proteins were visualized with an ECL detection system (Amersham Biosciences).

Preparation and transfection of siRNAs

The annealed double-stranded siRNAs listed below were obtained from Dharmacon, and were used to decrease expression of caspase-4. Caspase-4 siRNA-a: 5′-AAGUGGCCUCUUCACAGUCAUdTdT-3′ (sense), 5′-AAAUGACUGUGAAGAGGCCACdTdT-3′ (antisense); caspase-4 siRNA-b: 5′-AAGAUUUCCUCACUGGUGUUUdTdT-3′ (sense), 5′-AAAAACACCAGTGAGGAAATCdTdT-3′ (antisense). For control, siRNA to GFP was used. GFP siRNA: 5′-P-GGCUACGUCCAGGAGCGCACC-3′ (sense), 5′-P-UGCGCUCCUGGACGUAGCCUU-3′ (antisense). These sequences were not significantly homologous to genes other than caspase-4 or GFP by BLAST search (NCBI). SK-N-SH cells were transfected at 50% confluence in 24-well plastic plates with 1.0 μg of each of the above siRNAs using Transmessenger transfection reagent (QIAGEN) according to the manufacturer's protocol. Transfected cells were incubated at 37°C for 60 h without changing the medium. siRNAs were introduced into HeLa cells by electroporation three times with 48-h intervals using Amaxa system according to the manufacturer's protocol. Efficiency of RNAi was measured by immunocytochemical analysis and Western blot analysis using anti–caspase-4 antibody or anti–β-actin antibody.

Cell viability assay

SK-N-SH cells or HeLa cells overexpressing Bcl-2 and Bcl-x_L, or transfected with siRNAs as above were treated with various reagents as indicated. When using $A\beta_{1\text{-}40}$, it was preincubated for 1 wk at 37°C to establish its cytotoxicity. Cell death was assessed on the basis of cellular morpho-

logical changes observed by phase-contrast microscopy or from nuclear morphological changes detected by fluorescence microscopy after staining the cells with 10 μM Hoechst 33342. At least 500 cells were counted, and the data was expressed as the mean ± SEM from three independent experiments and P values were calculated by *t* test. The MTS ([3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]) assay was also performed to evaluate cell viability. After treatment with apoptosis-inducing reagents, cells were coincubated with MTS solution (Promega) for 1 h at 37°C. The amount of reduced MTS released from the viable cells was quantified by measuring the absorbance at 490 nm using a spectrophotometer. Results were expressed as a ratio (%) of dead cells after treatments to those in control as above.

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References

Alnemri, E.S., D.J. Livingston, D.W. Nicholson, G. Salvesen, N.A. Thornberry, W.W. Wong, and J. Yuan. 1996. Human ICE/CED-3 protease nomenclature. *Cell.* 87:171.

Bonifacino, J.S., and A.M. Weissman. 1998. Ubiquitin and the control of protein fate in the secretory and endocytic pathways. *Annu. Rev. Cell Dev. Biol.* 14: 19–57.

Brewer, J.W., J.L. Cleveland, and L.M. Hendershot. 1997. A pathway distinct from the mammalian unfolded protein response regulates expression of endoplasmic reticulum chaperones in non-stressed cells. *EMBO J.* 16:7207–7216.

Distelhorst, C.W., M. Lam, and T.S. McCormick. 1996. Bcl-2 inhibits hydrogen peroxide-induced ER Ca^{2+} pool depletion. *Oncogene.* 12:2051–2055.

Fernandes-Alnemri, T., R.C. Armstrong, J. Krebs, S.M. Srinivasula, L. Wang, F. Bullrich, L.C. Fritz, J.A. Trapani, K.J. Tomaselli, G. Litwack, and E.S. Alnemri. 1996. In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc. Natl. Acad. Sci. USA.* 93:7464–7469.

Ferri, K.F., and G. Kroemer. 2001. Organelle-specific initiation of cell death pathways. *Nat. Cell Biol.* 3:E255–E263.

Fischer, H., U. Koenig, L. Eckhart, and E. Tschachler. 2002. Human caspase 12 has acquired deleterious mutations. *Biochem. Biophys. Res. Commun.* 293: 722–772.

Forman, M.S., V.M. Lee, and J.Q. Trojanowski. 2003. 'Unfolding' pathways in neurodegenerative disease. *Trends Neurosci.* 26:407–410.

Foyouzi-Youssefi, R., S. Arnaudeau, C. Borner, W.L. Kelley, J. Tschopp, D.P. Lew, N. Demaurex, and K.H. Krause. 2000. Bcl-2 decreases the free Ca^{2+} concentration within the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA.* 97:5723–5728.

Harding, H.P., Y. Zhang, and D. Ron. 1999. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature.* 397:271–274.

Haviv, R., L. Lindenboim, J. Yuan, and R. Stein. 1998. Need for caspase-2 in apoptosis of growth-factor-deprived PC12 cells. *J. Neurosci. Res.* 52:491–497.

He, H., M. Lam, T.S. McCormick, and C.W. Distelhorst. 1997. Maintenance of calcium homeostasis in the endoplasmic reticulum by Bcl-2. *J. Cell Biol.* 138:1219–1228.

Imai, Y., M. Soda, and R. Takahashi. 2000. Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. *J. Biol. Chem.* 275:35661–35664.

Imai, Y., M. Soda, H. Inoue, N. Hattori, Y. Mizuno, and R. Takahashi. 2001. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell.* 105:891–902.

Imaizumi, K., K. Miyoshi, T. Katayama, T. Yoneda, M. Taniguchi, T. Kudo, and M. Tohyama. 2001. The unfolded protein response and Alzheimer's disease. *Biochim. Biophys. Acta.* 1536:85–96.

Iwahashi, H., Y. Eguchi, N. Yasuhara, T. Hanafusa, Y. Matsuzawa, and Y. Tsujimoto. 1997. Synergistic anti-apoptotic activity between Bcl-2 and SMN implicated in spinal muscular atrophy. *Nature.* 390:413–417.

Kamens, J., M. Paskind, M. Hugunin, R.V. Talanian, H. Allen, D. Banach, N. Bump, M. Hackett, C.G. Johnston, P. Li, et al. 1995. Identification and characterization of ICH-2, a novel member of the interleukin-1 beta-converting enzyme family of cysteine proteases. *J. Biol. Chem.* 270:15250–15256.

Katayama, T., K. Imaizumi, N. Sato, K. Miyoshi, T. Kudo, J. Hitomi, T. Morihara, T. Yoneda, F. Gomi, Y. Mori, et al. 1999. Presenilin-1 mutations downregulate the signaling pathway of the unfolded-protein response. *Nat. Cell Biol.* 1:479–485.

Kaufman, R.J. 2002. Orchestrating the unfolded protein response in health and disease. *J. Clin. Invest.* 110:1389–1398.

Kluck, R.M., E. Bossy-Wetzel, D.R. Green, and D.D. Newmeyer. 1997. The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science.* 275:1132–1136.

Kozutsumi, Y., M. Segal, K. Normington, M.J. Gething, and J. Sambrook. 1988. The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. *Nature.* 332:462–464.

Li, P., D. Nijhawan, I. Budihardjo, S.M. Srinivasula, M. Ahmad, E.S. Alnemri, and X. Wang. 1997. Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell.* 91: 479–489.

Lin, X.Y., M.S. Choi, and A.G. Porter. 2000. Expression analysis of the human caspase-1 subfamily reveals specific regulation of the CASP5 gene by lipopolysaccharide and interferon-gamma. *J. Biol. Chem.* 275:39920–39926.

Martinon, F., K. Burns, and J. Tschopp. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol. Cell.* 10:417–426.

Miyake, S., T. Yamashita, M. Taniguchi, M. Tamatani, K. Sato, and M. Tohyama. 2002. Identification and characterization of a novel mitochondrial tricarboxylate carrier. *Biochem. Biophys. Res. Commun.* 295:463–468.

Muzio, M., A.M. Chinnaiyan, F.C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J.D. Bretz, M. Zhang, R. Gentz, et al. 1996. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell.* 85:817–827.

Nakagawa, T., and J. Yuan. 2000. Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J. Cell Biol.* 150:887–894.

Nakagawa, T., H. Zhu, N. Morishima, E. Li, J. Xu, B.A. Yankner, and J. Yuan. 2000. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature.* 403:98–103.

Nishitoh, H., A. Matsuzawa, K. Tobiume, K. Saegusa, K. Takeda, K. Inoue, S. Hori, A. Kakizuka, and H. Ichijo. 2002. ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev.* 16:1345–1355.

Oyadomari, S., E. Araki, and M. Mori. 2002. Endoplasmic reticulum stress-mediated apoptosis in pancreatic beta-cells. *Apoptosis.* 7:335–345.

Pinton, P., D. Ferrari, P. Magalhaes, K. Schulze-Osthoff, F. Di Virgilio, T. Pozzan, and R. Rizzuto. 2000. Reduced loading of intracellular $Ca^{(2+)}$ stores and downregulation of capacitative $Ca^{(2+)}$ influx in Bcl-2–overexpressing cells. *J. Cell Biol.* 148:857–862.

Rao, R.V., E. Hermel, S. Castro-Obregon, G. del Rio, L.M. Ellerby, H.M. Ellerby, and D.E. Bredesen. 2001. Coupling endoplasmic reticulum stress to the cell death program. Mechanism of caspase activation. *J. Biol. Chem.* 276:33869–33874.

Salvesen, G.S., and V.M. Dixit. 1997. Caspases: intracellular signaling by proteolysis. *Cell.* 91:443–446.

Sato, N., K. Imaizumi, T. Manabe, M. Taniguchi, J. Hitomi, T. Katayama, T. Yoneda, T. Morihara, Y. Yasuda, T. Takagi, et al. 2001. Increased production of beta-amyloid and vulnerability to endoplasmic reticulum stress by an aberrant spliced form of presenilin 2. *J. Biol. Chem.* 276:2108–2114.

Scaffidi, C., S. Fulda, A. Srinivasan, C. Friesen, F. Li, K.J. Tomaselli, K.M. Debatin, P.H. Krammer, and M.E. Peter. 1998. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J.* 17:1675–1687.

Scorrano, L., S.A. Oakes, J.T. Opferman, E.H. Cheng, M.D. Sorcinelli, T. Pozzan, and S.J. Korsmeyer. 2003. BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis. *Science.* 300:135–139.

Selkoe, D.J. 1986. Altered structural proteins in plaques and tangles: what do they tell us about the biology of Alzheimer's disease? *Neurobiol. Aging.* 7:425–432.

Tagami, S., Y. Eguchi, M. Kinoshita, M. Takeda, and Y. Tsujimoto. 2000. A novel protein, RTN-XS, interacts with both Bcl-XL and Bcl-2 on endoplasmic reticulum and reduces their anti-apoptotic activity. *Oncogene.* 19:5736–5746.

Tamatani, M., T. Matsuyama, A. Yamaguchi, N. Mitsuda, Y. Tsukamoto, M. Taniguchi, Y.H. Che, K. Ozawa, O. Hori, H. Nishimura, et al. 2001. ORP150 protects against hypoxia/ischemia-induced neuronal death. *Nat. Med.* 7:317–323.

Thornberry, N.A., and Y. Lazebnik. 1998. Caspases: enemies within. *Science.* 281: 1312–1316.

Travers, K.J., C.K. Patil, L. Wodicka, D.J. Lockhart, J.S. Weissman, and P. Walter. 2000. Functional and genomic analyses reveal an essential coordina-

tion between the unfolded protein response and ER-associated degradation. *Cell.* 101:249–258.

Troy, C.M., S.A. Rabacchi, W.J. Friedman, T.F. Frappier, K. Brown, and M.L. Shelanski. 2000. Caspase-2 mediates neuronal cell death induced by beta-amyloid. *J. Neurosci.* 20:1386–1392.

Urano, F., X. Wang, A. Bertolotti, Y. Zhang, P. Chung, H.P. Harding, and D. Ron. 2000. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science.* 287:664–666.

Wang, S., M. Miura, Z. Jung, H. Zhu, V. Gagliardini, L. Shi, A.H. Greenberg, and J. Yuan. 1996. Identification and characterization of Ich-3, a member of the interleukin-1beta converting enzyme (ICE)/Ced-3 family and an upstream regulator of ICE. *J. Biol. Chem.* 271:20580–20587.

Wang, S., M. Miura, Y.K. Jung, H. Zhu, E. Li, and J. Yuan. 1998. Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. *Cell.* 92:501–509.

Wigley, W.C., R.P. Fabunmi, M.G. Lee, C.R. Marino, S. Muallem, G.N. DeMartino, and P.J. Thomas. 1999. Dynamic association of proteasomal machinery with the centrosome. *J. Cell Biol.* 145:481–490.

Yang, J., X. Liu, K. Bhalla, C.N. Kim, A.M. Ibrado, J. Cai, T.I. Peng, D.P. Jones, and X. Wang. 1997. Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. *Science.* 275:1129–1132.

Yankner, B.A., L.R. Dawes, S. Fisher, L. Villa-Komaroff, M.L. Oster-Granite, and R.L. Neve. 1989. Neurotoxicity of a fragment of the amyloid precursor associated with Alzheimer's disease. *Science.* 245:417–420.

Yoneda, T., K. Imaizumi, K. Oono, D. Yui, F. Gomi, T. Katayama, and M. Tohyama. 2001. Activation of caspase-12, an endoplastic reticulum (ER) resident caspase, through tumor necrosis factor receptor-associated factor 2-dependent mechanism in response to the ER stress. *J. Biol. Chem.* 276:13935–13940.

Zinszner, H., M. Kuroda, X. Wang, N. Batchvarova, R.T. Lightfoot, H. Remotti, J.L. Stevens, and D. Ron. 1998. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev.* 12:982–995.

Zong, W.X., C. Li, G. Hatzivassiliou, T. Lindsten, Q.C. Yu, J. Yuan, and C.B. Thompson. 2003. Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. *J. Cell Biol.* 162:59–69.

Zou, H., W.J. Henzel, X. Liu, A. Lutschg, and X. Wang. 1997. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome *c*-dependent activation of caspase-3. *Cell.* 90:405–413.