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 $A\beta_{1-42}$ toxicity (Troy et al., 2000). Thus $A\beta_{1-42}$-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, $A\beta_{25-35}$ and $A\beta_{1-40}$, and their reverse peptides, $A\beta_{35-25}$ and $A\beta_{40-1}$, 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 $A\beta_{1-40}$, 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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