A characteristic of many neurodegenerative diseases, including Parkinson’s disease and amyotrophic lateral sclerosis, is neuronal cell death with apoptotic morphological changes (1). Apoptotic cell death pathways can be triggered by either an extrinsic factors pathway triggered by death receptors such as Fas or by an intrinsic pathway mediated by mitochondria (2). Recently, ER stress was also shown to trigger neuronal apoptosis that proceeds via an alternative intrinsic pathway in various neurodegenerative diseases (3, 4). Most of the apoptotic morphological changes result from the activations of a set of cysteine proteases, a large protein family known as the caspases. All known caspases possess an active-site cysteine and cleave substrates at Asp-Xxx bonds (5). In fact, the central executioner procaspase-3 is cleaved at Asp-28 and Asp-175 to active caspase-3 by caspase-9, which is cleaved at Asp-315 to the active form when assembled to form an apoptosome with Apaf-1 and cytochrome c (6). Moreover, the activation of caspase-9 is also achieved via a feedback amplification loop in which caspase-3 processes procaspase-9 at Asp-330 (7).

Caspase-4 is a member of the caspase-1 family, known as inflammatory caspases, and localized in the endoplasmic reticulum (ER). Recently, some ER stress inducers such as tunicamycin and thapsigargin have been found to induce caspase-4 mRNA expression and protein activation (8). Moreover, cleavage of caspase-4 is not affected by overexpression of Bcl-2, which prevents signal transduction on the mitochondria, implying that caspase-4 is primarily activated in ER stress–induced apoptosis (9). Although the studies with chemical inhibitors and/or small interference RNA show that activations of both caspase-4 and caspase-9 are required for the prosecution process of ER stress–induced apoptosis, it is unclear the interaction between caspase-4 and caspase-9 in ER stress–induced apoptosis. In this study, we investigated the interrelationship between caspase-4 and caspase-9 in ER stress–induced apoptosis in human neuroblastoma SH-SY5Y cells.

Recombinant human caspases-4, tunicamycin, z-LEHD-fmk, and LEVD-CHO were obtained from Calbiochem (Darmstadt, Germany). Recombinant human procaspase-9 was purchased from Alexis (Lausanne, Switzerland). The following antibodies were obtained from the indicated sources: antibodies against caspases-9 (MBL, Nagoya), cytochrome c (BD Biosciences Pharmingen, San Diego, CA, USA), poly-ADP-ribose polymerase (PARP) and caspases-4 (Santa Cruz Biotech-
technology, Santa Cruz, CA, USA), and caspases-3 (Cell Signaling Technology, Boston, MA, USA). Plasmid (pET23b-Casp9-His) containing WT procaspase-9 was purchased from Addgene (Cambridge, MA, USA). Hoechst33342 was purchased from Sigma (St. Louis, MO, USA). Unless stated otherwise, all chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Wako, Osaka). Human neuroblastoma SH-SY5Y cells (Riken Cell Bank, Tsukuba) were grown in RPMI1640 medium (Sigma, Steinheim, Germany) at 37°C with 5% CO₂.

**Fig. 1.** Activation of caspase-9 and caspase-3 in tunicamycin-induced apoptosis. A) SH-SY5Y cells were treated with 50 μM tunicamycin for the indicated times. The extent of apoptosis was determined by counting cells with apoptotic nuclei. The protein levels of active caspase-9 (B), active caspase-3 (C), and cytosolic cytochrome c (D) were analyzed by Western blotting. E, F) Cells were incubated for 30 min with the caspase-9 inhibitor z-LEHD-fmk at the indicated concentrations before treatment with 50 μM tunicamycin for 24 h. Data represent the mean ± S.E.M. (n = 4). **P < 0.01 vs. tunicamycin (0 h), ††P < 0.01 vs. control, **P < 0.01 vs. tunicamycin (50 μM).
CO₂, as described previously (10).

To estimate the extent of apoptosis, we counted the number of cells with apoptotic nuclei stained with Hoechst33342, and determined the proportion of PARP molecules by Western blotting analysis. Cytosolic fractions were prepared according to a method described by Liu and Wang (11). Briefly, the cells were suspended in 20 mM HEPES buffer (pH 7.4) containing 10 mM KCl, 1.5 mM MgCl₂, 250 mM sucrose, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 1 mM PMSF, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 2 μg/ml aprotinin and then disrupted by freezing and thawing. The cell suspension was centrifuged at 100,000 × g for 30 min and the supernatant was used as the cytosolic fraction. Cells were lysed with a buffer of the following composition: 25 mM Tris-HCl, 5% glycerol, 1% SDS, 0.04% bromophenol blue, and 2% 2-mercaptoethanol, pH 6.8. The cell lysates (10 μg for PARP, 20 μg for caspases) or the cytosol fraction (10 μg) was separated by SDS-PAGE under reducing conditions and immunoblotted with appropriate antibodies, as described previously (12). We introduced mutations into the procaspase-9 element of pET23b-Casp9-His using the PrimeSTAR mutagenesis kit (Takara Bio, Shiga) and produced recombinant WT and mutant procaspase-9 using the TNT T7 Quick Coupled Translation/Transcription System (Promega, Madison, WI, USA). The integrity of all constructs was confirmed by DNA sequencing. One-way ANOVA was used to examine the differences between group means, followed by post-hoc analysis (Tukey test).

The blockade of N-glycosylation by tunicamycin elicits ER stress responses and induces caspase-mediated apoptosis in a variety of cultured cells, including neurons (13, 14). We first examined the effect of tunicamycin on nuclear morphology in human neuroblastoma SH-SY5Y cells. Treatment with tunicamycin increased the cells with apoptotic nuclear morphological change in a time-dependent manner (Fig. 1A). Tunicamycin also increased the levels of both active caspase-9 and active caspase-3 (Fig. 1: B and C) in a time-dependent manner. The content of cytochrome c in the cytosolic fractions was increased from 12 h after the treatment (Fig. 1D). Next, we examined the effect of caspase-9 inhibitor on tunicamycin-induced apoptosis. The caspase-9 inhibitor z-LEHD-fmk suppressed tunicamycin-induced apoptosis (Fig. 1E). Z-LEHD-fmk also suppressed the cleavage of PARP, which is a substrate for caspase-3, induced by tunicamycin (Fig. 1F).

Figure 2 shows the involvement of caspase-4 in tunicamycin-induced apoptosis. Treatment of SH-SY5Y cells with tunicamycin resulted in the transient increase of active caspase-4 protein level (Fig. 2A). The active caspase-4 level was increased from 6 h after the treatment and was maximal at 9-h after. The induction of active caspase-4 was earlier than that of active caspase-9. The caspase-4 inhibitor LEVD-CHO suppressed both apoptosis and cleavage of PARP induced by tunicamycin (Fig. 2: B and C). To reveal the interaction between caspase-4 and caspase-9, we examined whether caspase-4...
inhibitor LEVD-CHO suppressed the activation of caspase-9. LEVD-CHO dose-dependently suppressed tunicamycin-induced increase in the level of active caspase-9 (Fig. 3A), indicating that caspase-4 acts upstream of caspase-9 in tunicamycin-induced apoptosis in SH-SY5Y cells. We further examined whether caspase-4 directly activated caspase-9 by using human recombinant caspases. Western blot analysis revealed that the recombinant active caspase-4 cleaved the recombinant procaspase-9 (Fig. 3B). To reveal the processing site of procaspase-9 cleaved by caspase-4, the mutants of caspase-9 were generated by mutating amino acid residues 315 and 330 from aspartate into alanine (D315A and D330A). Recombinant caspase-4 cleaved the D330A mutant, but not the D315A mutant.

The major findings of the present study are that caspase-4 directly activates caspase-9 and that the processing of procaspase-9 by caspase-4 occurs at Asp-315. In the present study, the caspase-9 activation was earlier than cytochrome c release into the cytosol, and the activation of caspase-4 was earlier than the caspase-9 activation. In addition, the caspase-4 inhibitor LEVD-CHO inhibited tunicamycin-induced activation of caspase-9.

Procaspase-9 is cleaved into the active form of either 35 or 37 kDa depending on whether the cleavage site is Asp-315 or Asp-330. The cytochrome c / Apaf-1 complex (apoptosome)-triggered processing of procaspase-9 produces the p35 form via cleavage at Asp-315, and active capase-3 cleaves procaspase-9 at Asp-330 to produce p37 form (15). In the present study, human recombinant active caspase-4 cleaved recombinant procaspase-9 into a single form (Fig. 3B). In addition, the recombinant active caspase-4 could not process the D315A mutant of procaspase-9 (Fig. 3B). These results indicate that active caspase-4 can process procaspase-9 at Asp-315, the same cleavage site as the apoptosome, to produce active caspase-9.

Caspase-4 plays critical roles in ER stress–induced apoptosis in human retinal pigment epithelial cells (8), human carcinoma HeLa cells, and human neuroblastoma SK-N-SH cells (9). Consistent with these reports, in the present study, the caspase-4 inhibitor LEVD-CHO suppressed tunicamycin-induced apoptosis in SH-SY5Y cells (Fig. 2B). In addition, LEVD-CHO inhibited tunicamycin-induced cleavage of PARP (Fig. 2C). These results indicate that tunicamycin induces apoptosis in SH-SY5Y cells through the activation of caspase-4.

In conclusion, this study reveals a new apoptotic pathway in which caspase-4 directly activates caspase-9. The activation of caspases-4 may play an important role in the initiation of ER stress–induced neuronal apoptosis.

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References

4. Kim SJ, Zhang Z, Hitomi E, Lee YC, Mukherjee AB. Endoplasmic reticulum stress-induced caspase-4 activation mediates...