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# Apoptosis induced by endoplasmic reticulum stress depends on activation of caspase-3 via caspase-12

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**Abstract**

Recently, endoplasmic reticulum (ER) dysfunction has been implicated in neuronal death in patients with Alzheimer's disease. Treatment of human neuroblastoma cells with ER stress inducers causes apoptotic death. We confirmed that ER stress inducers specifically targeted the ER to cause apoptotic morphological changes. We also found that caspase-3, and not caspase-9 (a known mitochondrial apoptotic mediator), was mainly activated by ER stress. We generated the neuroblastoma cells that stably expressed caspase-12 and analyzed its influence on caspase-3 activation and vulnerability to ER stress. Cells expressing caspase-12 were more vulnerable to ER stress than cells expressing the empty vector, concomitant with increased activation of caspase-3. These findings suggested that activation of ER-resident caspase-12 indirectly activates cytoplasmic caspase-3 and might be important in ER stress-induced neuronal apoptosis.


*Keywords:* SK-N-SH neuroblastoma cells; Endoplasmic reticulum stress; Apoptosis; Caspase-12; Caspase-3

The endoplasmic reticulum (ER) is an organelle that ensures correct protein folding and assembly by expressing numerous molecular chaperones [3,7]. Under various conditions, such as glucose starvation, disturbance of intracellular calcium homeostasis, inhibition of protein glycosylation, or exposure to free radicals, unfolded proteins accumulate in the ER lumen, a process called ER stress. Excessive ER stress induces cell death [6,17]. Neuronal death in some neurodegenerative disorders, especially Alzheimer's disease (AD), is reported to be due to ER dysfunction. Familial AD-linked presenilin-1 mutants or presenilin-2 splice variants which are expressed in sporadic AD brains, and increase neuronal vulnerability to ER stress [5,13,14]. Thus, the ER may be important for regulating intracellular apoptotic signals in neurons, but the mechanisms of ER stress-induced cell death are poorly understood.

Caspase-12, one of the caspase family of proteases, is localized to the ER and is specifically activated by ER stress [10], with several possible mechanisms being suggested [9,12,18]. Caspase-12-deficient cells are resistant to ER stress, but not non-ER stress [10], and such evidence suggests that caspase-12 is a major mediator of ER-stress-induced apoptosis.

We found that activation of ER resident caspase-12 causes activation of cytoplasmic caspase-3, and not mitochondria-related caspase-9, during ER stress-induced apoptosis.

When cells die from ER stress, it is believed that apoptosis occurs, but whether these cells show typical morphological features of apoptosis is unclear. Therefore, we analyzed the morphological and biochemical changes during cell death after ER stress in SK-N-SH, human neuroblastoma cells.

*Abbreviations:* ER, endoplasmic reticulum; AD, Alzheimer's disease; Tm, tunicamycin; Tg, thapsigargin; AMC, 7-amino-4-methylcoumarin; LDH, lactate dehydrogenase.

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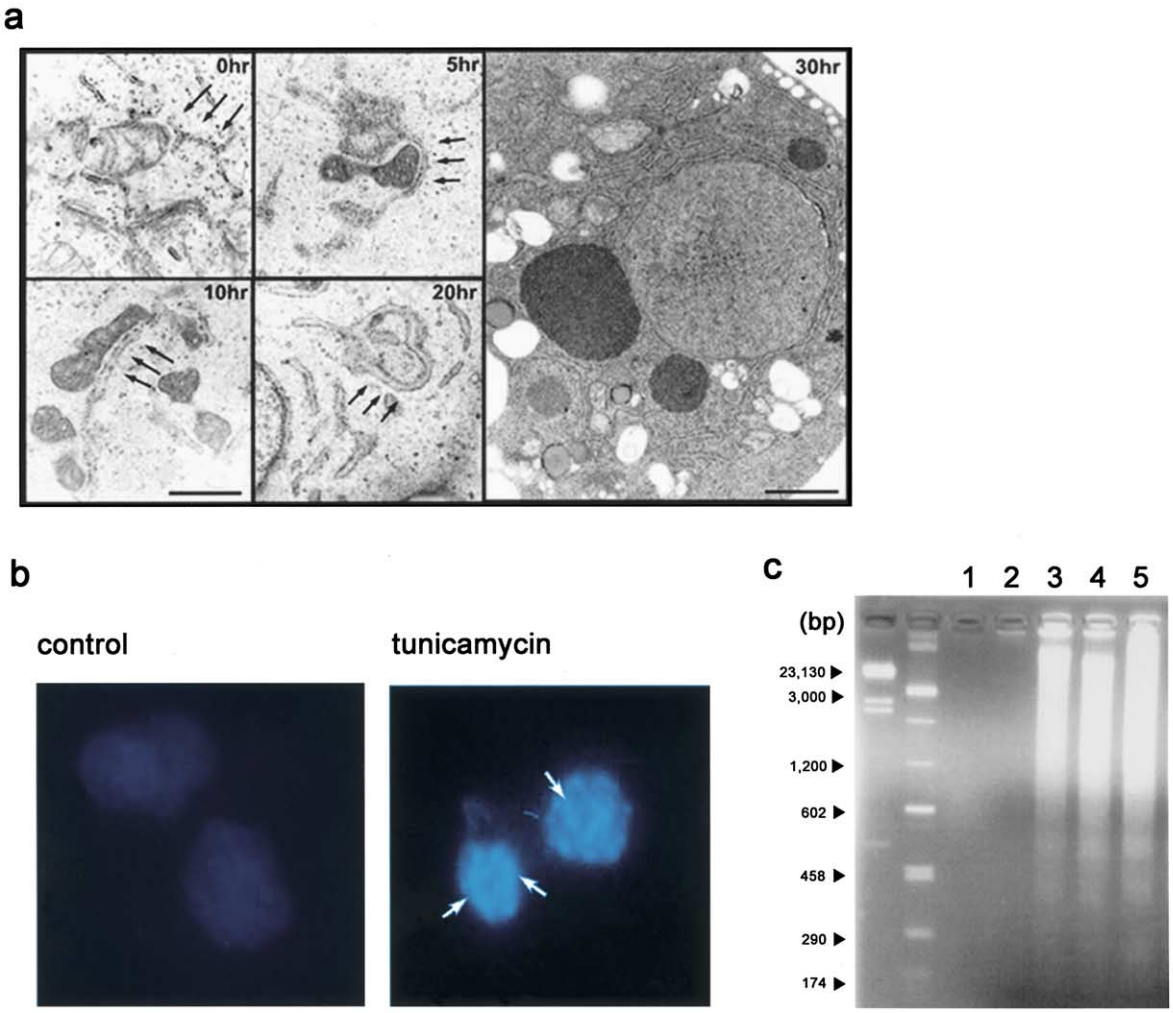


Fig. 1. Morphological and biochemical features of apoptosis induced by ER stress. (a) Electron microscopy shows ER luminal swelling (black arrow), detachment of ribosomes from rough ER, and nuclear fragmentation of SK-N-SH cells after treatment with an ER stress inducer, 1.0 μg/ml tunicamycin (Tm). ER luminal swelling occurred before nuclear fragmentation Scale bars represent 1 μm. (b) Hoechst 33258 staining of cells with or without exposure to 1.0 μg/ml Tm for 24 h. White arrows indicate chromatin condensation. (c) DNA laddering in cells treated with 1.0 μg/ml Tm (lane 3) and 0.5 μM Tg (lane 4) for 24 h or 0.1 μM staurosporine (lane 5) for 8 h. Data are representative results from three separate experiments.

As shown in Fig. 1, chronic ER stress (30 h of exposure to 1 μg/ml tunicamycin (Tm), a N-linked glycosylation inhibitor) caused apoptotic morphological changes, including nuclear fragmentation, chromatin condensation, and cell shrinkage. Interestingly, prior to these changes, swelling of the ER lumen and dissociation of ribosomes from rough ER were observed by electron microscopy.

These studies indicated that ER stress-induced cell death was a type of apoptosis.

Caspases are critical mediators of apoptosis in mammalian cells [16], so we measured several caspases activities using synthetic fluorometric substrates (50 μM Ac-YVAD-AMC, Ac-DEVD-AMC, Ac-VEID-AMC, Ac-IETD-AMC, and Ac-LEHD-AMC for caspase-1, caspase-3, caspase-6, caspase-8, and caspase-9, respectively). After 24 h of treatment with 1 μg/ml Tm or 0.5 μM thapsigargin (Tg), an ER $Ca^{2+}$-ATPase inhibitor, caspase-3 activity was significantly higher than that of other caspases (Fig. 2a, upper). Activation of caspases requires cleavage of pro-caspases, e.g. pro-caspase-3 is cleaved into smaller subunits under apoptotic conditions [1]. To confirm the role of caspase-3 in ER stress-induced apoptosis, we assessed pro-caspase-3 cleavage in SK-N-SH cells treated with 1 μg/ml Tm by western blotting using an anti-caspase-3 antibody that detected both uncleaved and cleaved caspase-3 (Fig. 2a, bottom). Cleaved caspase-3 was observed under ER stress conditions. Next, we examined the effects of specific caspase inhibitors on apoptosis (Fig. 2b). After exposure to 1 μg/ml Tm with or without each caspase inhibitor (5 μM), cell death was quantified from LDH release into the medium. When cells were treated with both Tm and a caspase-3 inhibitor, death was significantly reduced, suggesting that caspase-3 was a major caspase activated by ER stress. Similar results were observed after treatment with Tg (Fig. 2b, right).

To clarify the link between activation of ER resident

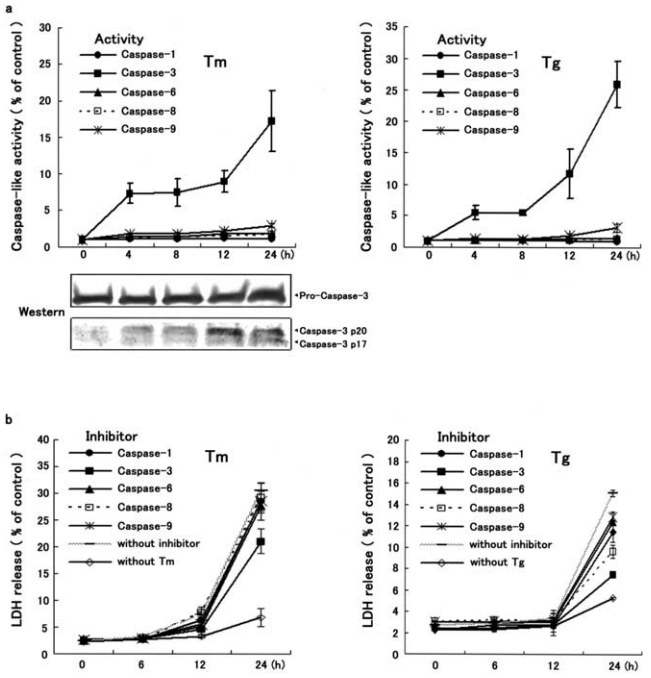


Fig. 2. Caspase-3 activation by ER stress. (a) SK-N-SH cell lysates (50 μg) treated with 1.0 μg/ml Tm or 0.5 μM Tg were incubated with 50 μM Ac-YVAD-AMC (caspase-1, closed circles), Ac-DEVD-AMC (caspase-3, closed squares), Ac-VEID-AMC (caspase-6, closed triangles), Ac-IETD-AMC (caspase-8, open squares), or Ac-LEHD-AMC (caspase-9, asterisks) fluorometric substrates at 37 °C for 30 min and caspase activity was measured using a spectrophotometer with excitation at 380 nm and emission at 460 nm (upper panel). Cytosol was immunoblotted with anti-caspase-3 recognizing both uncleaved and active forms (p20, p17) after treatment with 1.0 μg/ml Tm (bottom panel). (b) To confirm the effect of caspase-3 on death of SK-N-SH cells treated for 24 h with 1.0 μg/ml Tm and 5 μM of each caspase inhibitor [Ac-YVAD-CHO (caspase-1, closed circles), Ac-DEVD-CHO (caspase-3, closed squares), Ac-VEID-CHO (caspase-6, closed triangles), Ac-IETD-CHO (caspase-8, open squares), or Ac-LEHD-CHO (caspase-9, asterisks)], culture medium was collected to assay LDH. Data are mean values from four independent experiments.

caspase-12 and cytoplasmic caspase-3, we assessed caspase-3 activation in SK-N-SH cells with stable expression of caspase-12 or empty vector (mock). Caspase-3 activation was significantly greater in caspase-12-expressing cells than in mock-expressing cells after treatment with 1 μg/ml Tm for 10 h (Fig. 3a). Cleavage of pro-caspase-3 was also increased in caspase-12-expressing cells (Fig. 3a). To investigate whether increased caspase-3 activation in caspase-12-expressing cells was accompanied by vulnerability to ER stress, we compared LDH release by caspase-12- and mock-expressing cells after treatment with 1 μg/ml Tm. Overexpression of caspase-12 increased the vulnerability of cells to Tm (Fig. 3b). To determine whether activated caspase-12 could directly cleave pro-caspase-3, we performed an in vitro cleavage assay using recombinant active caspase-12 [8,10], but found no cleavage of in vitro-translated pro-caspase-3 by recombinant caspase-12 (data not shown). These results suggested that activated caspase-12 might indirectly activate cytoplasmic caspase-3 during ER stress.

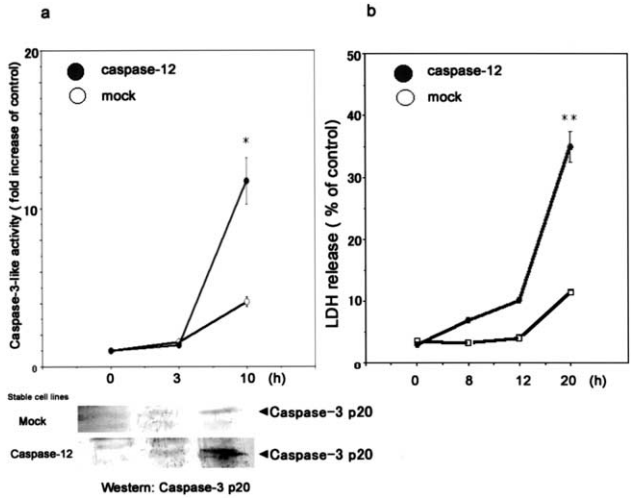


Fig. 3. Caspase-12 overexpressing neuroblastoma cells activate caspase-3 and potentiate vulnerability to ER stress. (a) Increase of caspase-3 activation in cells stably expressing caspase-12 compared with mock (empty vector)-expressing cells. SK-N-SH cells expressing caspase-12 (closed circles) or mock vector (open circles) were treated with 1.0 μg/ml Tm for 20 h and lysed with ICE buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA 1 mM EGTA, and 10 μM digitonin). Equal amounts of lysates (50 μg) were incubated with 50 μM Ac-DEVD-MCA at 37 °C for 30 min to measure caspase-3-like activity using a spectrophotometer with excitation at 380 nm and emission at 460 nm. (b) Vulnerability of caspase-12-overexpressing cells compared with mock-expressing cells. After treatment with 1.0 μg/ml Tm for 20 h, culture medium was harvested for LDH assay by the manufacturer's protocol (KYOKUTO, Japan). Data are the mean of four independent experiments.

This study showed that activation of exogenous murine caspase-12 led to activation of cytoplasmic caspase-3, rather than caspase-9, in ER stress-induced apoptosis. We also showed by electron microscopy that ER stress inducers cause ER swelling prior to mitochondrial disruption. These findings suggested that cross-talk between these ER and cytoplasmic caspases is important for ER stress-induced apoptosis in SK-N-SH cells, rather than ER-mitochondrial cross-talk [4,15]. This possibility was recently supported by Rao et al. [11]. There have been several reports on the molecular mechanism of caspase-12 activation [9,12,18], so caspase-12 may play a role in apoptosis after ER stress.

Human caspase-12 has not been cloned yet and there is controversy about its expression [2,12]. Consequently, we investigated murine caspase-12 as a substitute for endogenous human caspase-12-like protease. Future isolation and functional analysis of human caspase-12 may lead to better understanding of ER stress-induced apoptosis in human cells.

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