Endoplasmic Reticulum Stress Induces Apoptosis by an Apoptosome-dependent but Caspase 12-independent Mechanism*

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The endoplasmic reticulum (ER) is the cellular site of polypeptide folding and modification. When these processes are hampered, an unfolded protein response (UPR) is activated. If the damage is too broad, the mammalian UPR launches the apoptotic program. As a consequence, mobilization of ER calcium stores sensitizes mitochondria to direct proapoptotic stimuli. We make use of a mouse Apaf1-deficient cell system of proneural origin to understand the roles played in this context by the apoptosome, the most studied apoptotic machinery along the mitochondrial pathway of death. We show here that in the absence of the apoptosome ER stress induces cytochrome c release from the mitochondria but that apoptosis cannot occur. Under these circumstances, Grp78/BiP and GADD153/CHOP, both hallmarks of UPR, are canonically up-regulated, and calcium is properly released from ER stores. We also demonstrate that caspase 12, a protease until now believed to play a central role in the initiation of ER stress-induced cell death in the mouse system, is dispensable for the mitochondrial pathway of death to take place.

Stress conditions interfering with the homeostasis of the ER initiate diverse signaling responses, resulting in a decreased rate of protein translation so as to prevent further accumulation of unfolded proteins (1). Simultaneously, transcription factors are activated to induce the expression of ER-resident chaperones so as to deal with accumulated protein aggregates. In addition, the ER-specific protein-degrading apparatus also becomes activated and eliminates denatured proteins. Comprehensive, this ER response to stress is named unfolded protein response (UPR) (2). When the accumulation of protein aggregates is overwhelming or their clean up is somehow hampered, the stress cannot be resolved, and the cell dies by apoptosis. In mammals, at least three ER transmembrane proteins (Ire1, ATF6, and PERK) respond to the accumulation of unfolded proteins in the lumen (2). Each of these three proteins has been shown to trigger the expression of proapoptotic genes when a proper ER internal protein balance cannot be restored (3). For its part, Ire1 has been shown to trigger apoptosis via TRAF2-mediated induction of caspase 12 oligomerization and cleavage (4). Caspases are cysteine-aspartate proteases responsible for the cleavage of cellular substrates in several apoptotic pathways (5). They can be divided into two groups, initiator caspases and executioner caspases. The executioner caspases, caspase 3 being the best characterized and probably the most conserved and effective among them, respond to direct activation by the initiator caspases. One of the best defined pathways of caspase-dependent death is promoted by the mitochondrion, via release of cytochrome c into the cytosol (6, 7). When this release occurs, a multimolecular complex is formed between the adapter molecule Apaf1 and the initiator caspase 9, namely the apoptosome, a potent trigger of cell death (6, 8). In a model suggested by Nakamura et al. (9), mitochondria are highly sensitive to Ca2+-release from ER upon UPR, this being achieved by two well-characterized types of channels, the inositol 1,4,5-triphosphate receptor and the Ryanodine receptor families (10, 11). In this model, cytosolic Ca2+ would be released from ER and would lead to the activation of several transcription factors, which in turn would induce cytochrome c release from mitochondria and apoptosome formation. Alternatively, privileged transport of Ca2+ between juxtaposed ER and mitochondrial membranes may also sensitize mitochondria to the proapoptotic effects of Bcl2 family members. Caspase 12 has been proposed as an initiator caspase and also as the key molecule in the death-driving force in ER stress (12). In fact, caspase 12 has been suggested as a direct activator of caspase 9, independently of cytochrome c release from mitochondria and apoptosome formation (13, 14).

An interesting issue to be clarified relates to the pathway(s) by which ER stress-mediated cell death occurs in the nervous system in physiological or pathological conditions. To analyze the possible involvement of the apoptosome-dependent pathway in these conditions, we used ETNA cells, a cellular model that we recently set up and that has the features of bona ﬁde neural precursors (15). These cells are derived from striatum primordia of wild-type (wt) or Apaf1+/−embryos (the latter are apoptosome-deficient (16, 17)) and are named ETNA−/+ and ETNA−/−, respectively.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Apaf1 expression plasmid was created by cloning of Apaf1 cDNA under control of the strong promoter CAGGs (CMV/β-actin, a kind gift of Dr. Miyazaki, Osaka, Japan). C9DN and C9wt expression plasmids were created by cloning of caspase 9DN

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4 The abbreviations used are: ER, endoplasmic reticulum; UPR, unfolded protein response; wt, wild-type; MEF, mouse embryonic fibroblast; BAPTA, 1,2-bis(O-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid, 4Na; PBS, phosphate-buffered saline; PARP, poly(ADP-ribose) polymerase; TN, tunicamycin; Z-VAD-fmk, benzoyloxycarbonyl-VAD-fluoromethyl ketone; AIF, apoptosis-inducing factor; PI, propidium iodide; DAPI, 4’,6-diamidino-2-phenylindole; DN, dominant negative; siRNA, small interfering RNA.

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FIGURE 1. Analysis of ER-stress-induced apoptosis in wt or apoptosome-deficient cells. a, ETNA+/+ and ETNA−/− cells were treated with TN at the indicated concentrations for 48 h, stained with propidium iodide (PI), and analyzed by flow cytometry. PI staining reveals the DNA content: The G1 and the G2/M peaks are labeled in the first panel. Percentages of apoptotic cells are indicated in each panel. The arrow points to the peak indicating cell growth arrest in G1 phase. b, quantitative analysis of data as reported in a. c, cells were treated...
cDNA and caspase 9wt cDNA (see below) under control of the CMV promoter in the mammalian expression vector pcDNA3 (Invitrogen) by using the EcoRI site of the pcDNA3 multiple cloning site. The hairpin expression vector, capable of expressing functional double-stranded siRNAs following transfection into eukaryotic cells, is being described elsewhere.\(^5\) To summarize, the BglII-Xhol fragment of the vector pcDNA3, which contains the CMV promoter, was replaced with a cassette that consists of a BglII-EcoRI fragment with the PolIII-specific promoter of the human H1 RNA gene (X16612) and an EcoRI-Xhol fragment with the stem-loop and a dT\(_6\) terminator. The presumed transcription start site is the first nucleotide after the EcoRI site. The stem-loops were always 23 bp in length and closed by an UUCG tetraloop. The 5\textquotesingle- part of the stem-loop was equal to the coding strand of the mRNA, and the 5\textquotesingle- part contained the respective antisense. The following regions of murine caspase 12 were selected: positions 1036–1058, 1071–1093, 1249–1271, and 1680–1702 (numbered as in GenBank\textsuperscript{TM} entry Y13090). The corresponding EcoRI-Xhol fragments were synthesized chemically (MWG, Milan) and subcloned into the stem-loop expression vector. The sequences were verified by dideoxy sequencing (Seqlab, Göttingen).

**Caspase 9 Site-directed Mutagenesis**—The caspase 9 dominant negative cDNA (C9DN) was created by site-directed mutagenesis of the caspase 9wt cDNA (C9wt) contained in the pDrive cloning vector (QIagen) by PCR using the following mutated oligonucleotides, (hdn) 5\textquotesingle- TGCGGCTGACCGAAGGTGACTTCTCTCGAG-3\textquotesingle- and (hdn) 5\textquotesingle- TTCTAGCGGCTGACCGAAGGTGACTGAG-3\textquotesingle- (the mutation C952G). The NruI fragment was cloned into the BglII site of pC9DN caspase 9wt cDNAs (1.5 \(\mu\)g of each plasmid) and with a plasmid carrying a gene for the hygromycin resistance (100 ng) using 5\textquotesingle- AAGCTTGGATGAAGAAGAG3\textquotesingle- and 5\textquotesingle- CTGCTCACCACCGTGCCTAAATCGGAAAG3\textquotesingle- primers (Invitrogen). The corresponding EcoRI fragment was amplified by PCR using the above primers. The PCR product was digested with EcoRI and XhoI and subcloned into pcDNA3, which contains the CMV promoter, to create pC9DN. The sequences were verified by dideoxy sequencing (Seqlab, Göttingen).

**Gene Transfer for Stable RNA Interference of Caspase 12**—The four hairpin expression vectors containing four different regions of murine caspase 12 for its RNA interference were introduced into cells by lipofection using Lipofectamine 2000 reagent. To summarize, ~5 \(\times\) 10\(^5\) cells containing 2 ml of the appropriate complete growth medium were seeded in a 35-mm dish and incubated at 33 \(^\circ\)C. The next day, 70–80% confluent cells were rinsed with serum-free and antibiotics-free OPTI-MEM (Invitrogen) and co-transfected with all four pcDNA3 hairpin expression vectors (1 \(\mu\)g of each plasmid) and with a plasmid carrying a gene for the hygromycin resistance (100 ng) using 5 \(\mu\)l of Lipofectamine. Cells were incubated at 33 \(^\circ\)C and after 4 h were allowed to recover by adding serum to the medium. 48 h after lipofection cells were split into four 90-mm Petri dishes. After growth without selection for a further day, medium containing 200 \(\mu\)g/ml hygromycin to select the resistant clones was applied. After 2 weeks colonies were picked and expanded for the examination of caspase 12 expression by Western blotting.

**Immunocytochemistry**—Cells were cultured in Petri dishes and fixed with 4% paraformaldehyde in PBS for 15 min. After permeabilization with 0.4% Triton X-100 in PBS for 15 min, cells were blocked with 2% horse serum in PBS and incubated for 1 h at 37 \(^\circ\)C with primary antibodies. We used an anti-cytochrome c mouse monoclonal antibody (clone 6H2.B4, BD Pharmingen), an anti-active-casp3 polyclonal antibody (Cell Signaling), and an anti-AIF rabbit polyclonal antibody (a generous gift of Guido Kroemer). Cells were then washed three times with blocking buffer and incubated for 1 h with labeled anti-mouse and anti-rabbit secondary antibodies (Alexa, Molecular Probes). After three washes in blocking buffer, cells were incubated with 25 \(\mu\)l SYTOX (Molecular Probes) and examined under a Zeiss LSM 510 Confocal Microscope. Fluorescence images were adjusted for brightness, contrast and color balance by using Adobe Photoshop 7.0. The TUNEL assay was performed by means of the kit from Promega according to the manufacturer’s instructions.

**Western Blot Analysis**—Cells were washed twice with PBS and scraped into radioimmune precipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl, pH 7.5) with freshly added protease inhibitors. After an incubation for 30 min on ice and a brief sonication, the lysate was centrifuged at 14,000 rpm for 10 min at 4 \(^\circ\)C, to remove the insoluble cell debris. 25 \(\mu\)g of total protein were separated by electrophoresis through SDS-polyacrylamide gels and blotted onto nitrocellulose. GADD153 and Grp78 were detected with monoclonal mouse-anti-GADD153 or anti-Grp78 antibodies (Santa Cruz) diluted 1:1000 and visualized with an affinity-purified anti mouse peroxidase conjugated IgG (Jackson Laboratories) diluted 1:10,000. A monoclonal mouse anti-\(\beta\)-tubulin (Sigma) diluted 1:1000 was used as a loading control. Caspase 3, 7, 9, 12, poly(ADP-ribose) polymerase (PARP), and cleaved PARP rabbit polyclonal antibodies (Cell Signaling) were used at 1:1000 dilution.

**RESULTS**

We have previously analyzed ETNA cells in standard and neurodegenerative death conditions, observing a peculiar resistance to cell death caused by the absence of the apoptosome (15). To cause ER stress, in the series of experiments we present here we applied three pharmacological agents to the ETNA cells, widely used death inducers that inhibit N-linked glycosy-
FIGURE 2. Visualization of apoptotic primary or cultured cells upon ER stress. a, cells were treated with TN 1.5 μg/ml for different times, stained with PI, and analyzed by flow cytometry; results are means ± S.D. of three independent determinations. b, SYTOX (green) and cytochrome c (red) staining of ETNA cells (upper panels) or primary cortical cells from wt and Apaf1−/− embryonic brains (lower panels) treated with TN (3 μg/ml) for 48 h. Arrows point to pycnotic nuclei of apoptosing cells. c, DAPI (blue) and TUNEL-positive (green) ETNA+/− or ETNA−/− cells treated with TN (3 μg/ml) for 48 h. Scale bars: 20 μm. d, left panel, Western blot detection of caspase 9 (C9) in ETNA+/− cells transiently overexpressing Casp9DN (DN) or Casp9wt (C9); right panel, Western blot analysis of PARP, cleaved PARP (Cl-PARP), and cleaved caspase 3 (Cl-C3) in ETNA+/− cells (Ctrl) and ETNA+/− cells transiently overexpressing Casp9DN untreated (UT) or treated with 3 μg/ml TN.
lulation (tunicamycin, TN), inhibit intracellular calcium pumps (thapsigargin), or block ER-to-Golgi transport (brefeldin A) (3). ETNA+/+ and ETNA−/− cells were cultured in the presence of increasing concentrations of TN (C9DN), which has previously been reported to act as a dominant negative, into ETNA−/− cells. When compared with cells transfected with wild type caspase 9, the C9DN mutant hampers apoptosis in ETNA−/− cells, as shown by reduction of caspase 3 and PARP processing (Fig. 2b, upper panels), or by the TUNEL method in ETNA−/− cells in marked contrast with wt cells (Fig. 2c). To verify that this resistance to death is a phenomenon also present in vivo, we extended this study to differentiated cells, using primary neurons dissected from the cortical primordia of mouse embryos devoid of Apaf1 and the corresponding wt littermates. Indeed, apoptosis did not occur in primary neurons upon induction by ER stress when the Apaf1 gene was inactivated (Fig. 2b, lower panels). To confirm a key role for a functional apoptosome in ER-induced apoptosis, we transiently transfected a catalytic mutant form of caspase 9 (C9DN), which has previously been reported to act as a dominant negative, into ETNA−/− cells. When compared with cells transfected with wt caspase 9, the C9DN mutant hampers apoptosis in ETNA−/− cells, as shown by reduction of caspase 3 and PARP processing (Fig. 2d) and lack of TUNEL positivity (not shown), analogously to what we have shown within ETNA−/− cells with the same stimuli.

To confirm the activation of apoptosis by UPR during the experiments performed with TN, we analyzed the expression levels of specific UPR markers such as GADD153/CHOP and Grp78/Bip, which are up-regulated by the UPR-receptors IRE1, ATF6, and PERK and which in turn may switch on the putative apoptotic cascade leading to caspases activation (18). Both factors were up-regulated, independently of the presence of the apoptosome, within a few hours from the application of the stimulus (Fig. 3).

Following the road to death downstream of ER stress, we used immunofluorescence to analyze the occurrence of cytochrome c release from mitochondria and the subsequent activation of an executioner caspase such as caspase 3 in ETNA+/+ and ETNA−/− cells. Upon ER stress induced by TN, brefeldin A, or thapsigargin (Fig. 4a, data not shown, respectively) cytochrome c is released from mitochondria independ-
chrome c release in this cell system upon TN-mediated ER stress. It has been reported that an executioner caspase, namely caspase 7, can mediate the activation of a signal traveling from the ER to the mitochondria as a consequence of death-inducing UPR (19). In a first experiment we inhibited caspase activity with the pan-caspase inhibitor Z-VAD-fmk. In this condition, upon TN death induction, cytochrome c is normally released from mitochondria, independently of caspase activity (Fig. 4c, arrows). Furthermore, the proapoptotic flavoprotein apoptosis-inducing factor (AIF) is not released from mitochondria together with cytochrome c after induction of ER stress using 40 h TN (Fig. 4c), as we previously reported for other death stimuli (15). This finding also supports a possible caspase-dependence of its nuclear translocation (20, 21). However, it should be mentioned that 72 h after the application of the stimulus, AIF disappears from some of the treated cells, probably because it is released in the cytosol and rapidly degraded. AIF release from mitochondria, however, does not always coincide with its translocation to the nucleus, it is not accompanied by an evident nuclear pycnosis nor an alternative cell death morphotype (Fig. 5).

Due to the fact that an Apaf1-independent intrinsic pathway of cell death has been previously described (13) in MEFs and to extend our findings to an additional, third cell system, we applied TN and thapsigargin stimuli (Fig. 6 and not shown, respectively) to MEFs dissected from Apaf1−/− embryos (e13.5) and wt littermates. Upon a 40-h-lasting ER-stress stimulus, mutant MEFs are TUNEL negative and do not show cleavage of caspase 3, caspase 7, and PARP, whereas wt MEFs succumb by massive cell death.

Because the release of Ca2+ from the ER and its concentration in the cytosol has been shown to be involved in cell death induction upon ER stress (22), we decided to analyze the role of Ca2+ in ETNA cell death by means of inhibitors of Ca2+ translocation or Ca2+ chelators (Fig. 7a). We used dantrolen, which is a strong inhibitor of Ca2+ release from ER, and BAPTA, which is a powerful Ca2+ chelator within the cytosol. By means of both drugs, we registered a significant decrease of apoptosis when ER stress was induced by TN (Fig. 7a). As a consequence of these results, we can conclude that in our cell system ER-stress-induced apoptosis depends on Ca2+ release from ER during UPR, as well as on the cytosolic Ca2+ concentration.

The widespread calpain proteases were also proposed as ER-localized mediators of mitochondria activation (23) or as direct death effectors by caspase 7 activation (19). For this reason, we analyzed calpain activation in ETNA cells upon ER stress by increasing concentrations of TN and using calpastatin as a calpain inhibitor (Fig. 7b). No difference in cell death rate could be observed upon TN induction independently of the use of calpastatin or of an unrelated peptide (as a specificity control). We can conclude that calpains are not the upstream key mediators of mitochondrial signaling or of caspase activity in this context.

The role of caspase 12 in humans may have been widely doubted (because of the absence of a reactive proenzyme), but in mice caspase 12 has been shown to be located at the cytosolic side of the ER membrane and to be actively cleaved in response to ER stress (24, 25). In fact, caspase 12 is the only known substrate to caspase 12 itself. In mouse, loss of caspase 12 has been shown to limit cell death upon TN induction, and experiments in mouse caspase 12−/− embryonic cortical neurons have been performed to prove its role in ER-stress-associated neurodegeneration (26). Because of the strong epistatic relationship we observed between ER stress and apoptosis in UPR-mediated cell death, we decided to verify the roles played by caspase 12 in our system. At first, we analyzed procaspase 12 cleavage by Western blot in ETNA wt and Apaf1-deficient cells. We were surprised to observe that in the latter, procaspase 12 accumulates strongly after 48 h of TN treatment instead of being cleaved and degraded (Fig. 8a). This suggests that caspase 12 activation occurs downstream of the apoptosome action.
and therefore precludes a crucial role in triggering cell death. To confirm our result, we utilized RNA interference, which enabled us to isolate several ETNA cell clones partially or almost completely depleted of caspase 12 (Fig. 8b), namely the ETNA/H11001/H11001/C12 cells. Among the chosen clones, numbers 4 and 21 did not express any detectable caspase 12 molecules, as revealed by Western blot. We therefore proceeded to analyze cell death occurrence and caspase activation in these clones upon TN-induced ER stress. In these systems the UPR transcriptional activity is activated (as described above, Fig. 8c), both caspase 7 and caspase 3 for their part are normally activated by cleavage upon TN induction, and apoptosis takes place normally (Fig. 8, d–f).

DISCUSSION

Overall, our results show that the apoptosome is a key complex in ER-stress-mediated apoptosis in at least three different murine cell types, such as embryo-derived neural precursor cells, embryonic primary cortical cells, and mouse embryonic fibroblasts. In the absence of the apoptosome (both by Apaf1 deficiency or expression of caspase 9 dominant negative) death cannot occur upon unresolved UPR by means of the execution pathways typical of apoptosis or by other alternative mechanisms, such as a caspase-independent and AIF-dependent pathway of cell demise. The fact that AIF is lost from mitochondria after a long exposure to the stress but does not irreversibly trigger cell death argues for a passive role of this molecule in this response. It is likely that mitochondria undergo in these conditions a general dysregulation that culminates in releasing factors with no direct consequences in activating death pathways. The evidence of cell survival in vitro upon persistence of death stimuli was described previously by us in the ETNA cell system (15). This finding was in line with the fact that Apaf1−/− brains undergo cell proliferation and differentiation upon an unexploited apoptosis induction and in absence of any alternative nonapoptotic death in vivo (15, 16). Admittedly, it remains to be unraveled on which basis cells are able to survive after release and degradation of cytochrome c (15) or AIF (this work), which should have as a logic consequence (or prerequisite) a severe damage of mitochondrial functionality. Furthermore we also propose that the apoptosome acts upstream of caspase 12 cleavage and also that caspase 12 activity is not required for ETNA cells to undergo ER-stress-mediated cell death. This evidence is particularly relevant regarding the proposed involvement of caspase 12 in the neuronal cell loss via ER stress induced by neurodegenerative stimuli. In fact, it has been proposed that ER-stress-dependent activation of caspase 12 might enhance Aβ-mediated cytotoxicity, a potential cause of Alzheimer disease, and so lead to its intracellular accumulation and/or increased secretion (26). This thesis has already been confuted in humans, where caspase 12 has been found to be truncated in Caucasian populations and uncleavable in Afro-American populations because of point mutations along its sequence (27). No relationship whatsoever between the incidence of Alzheimer disease and the occurrence of these mutations was found, ruling out caspase 12 defects from being involved in Alzheimer disease (27). We demonstrate here that

FIGURE 7. Involvement of calcium homeostasis in TN-induced apoptosis. a, ETNA+/− cells were treated with TN (3 μg/mL) for 48 h in the absence or presence of dantrolen (Dant, 1 μM) or BAPTA (5 μM), stained with PI, and analyzed by flow cytometry. The results reported are the means ± S.D. of three independent determinations. b, ETNA+/− cells were treated with TN at the indicated concentrations for 48 h in the absence or presence of calpastatin (CS) or a negative control (ur, unrelated peptide), stained with PI, and analyzed by flow cytometry.
ER stress induces cell death in mice through a caspase 12-independent pathway and we have previously shown that the apoptosome is absolutely required for Aβ-mediated apoptosis (15). Therefore, we strongly believe that in the mouse nervous system, as in humans, caspase 12 could play, at most, an executive downstream role in ER stress. These results are in line with the findings of Obeng and Boise (28) in a murine pro-B cell line or a human multiple myeloma line, obtained by means of modulating caspase 12 expression during ER stress.

In conclusion, we define here a preferential role for the apoptosome in ER-stress-mediated apoptosis in cells of proneural origin, primary striatum primordial cells and embryonic fibroblasts. We also show that the presence of high levels of Ca\(^{2+}\) in the cytosol after its release from ER is required for mitochondria to release cytochrome c and induce apoptosome formation. Regarding the role of caspase 12, our findings are in line with the observation that caspase 12 is a negligible mediator in several cell types in humans, being also in mouse a redundant and dispensable, possibly active, downstream executioner death protease.

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