ER stress-induced apoptosis and caspase-12 activation occurs downstream of mitochondrial apoptosis involving Apaf-1

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Summary
Accumulation of unfolded proteins induces endoplasmic reticulum (ER) stress. Excessive and prolonged stresses lead cells to apoptosis. However, the precise molecular mechanisms of ER stress-induced apoptosis have not been fully elucidated. We investigated the involvement of the apoptosome in ER stress-induced cell death pathway using mouse embryonic fibroblasts (MEFs) and mice deficient for Apaf-1. Apaf-1-deficient MEFs showed more resistance to ER stress-inducing reagents as compared with wild type cells. Despite comparable induction of ER stress in both wild type and Apaf-1-deficient cells, activation of caspase-3 was only observed in wild type, but not Apaf-1-deficient, MEFs. Under ER stress conditions, BAX translocated to mitochondria and cytochrome c was released from mitochondria. We also demonstrated that caspase-12 was processed downstream of Apaf-1 and caspase-3, and neither overexpression nor knockdown of caspase-12 affected susceptibility of the cells to ER stress-induced cell death. Furthermore, in the kidneys of Apaf-1-deficient mice, apoptosis induced by in vivo administration of tunicamycin was remarkably suppressed as compared with wild type mice. These data collectively demonstrated that Apaf-1 and the mitochondrial pathway of apoptosis play significant roles in ER stress-induced apoptosis.

Key words: Apoptosis, Apaf-1, ER stress, Mitochondria

Introduction
The endoplasmic reticulum (ER) is a factory responsible for the synthesis, initial post-translational modification, proper folding, and maturation of newly synthesized transmembrane and secretory proteins, as well as a regulator of intracellular calcium homeostasis. Various stresses, including expression of mutant proteins, viral infection, energy or nutrient deprivation, extreme environmental conditions, alterations in redox status or glycosylation status, or calcium release from the lumen of the ER, disrupt proper function of ER and cause so-called ER stress. The prototypic response of ER stress is the congestion of misfolded proteins. ER stress leads cells to activate self-protective mechanisms: (1) transcriptional up-regulation of ER chaperones and folding enzymes; (2) translational attenuation to limit further accumulation of misfolded proteins; and (3) ER-associated degradation (ERAD) which eliminates misfolded proteins from the ER. The current understanding is that three types of ER membrane receptors, ATF6, IRE1 and PERK may sense the stress in the ER, and eventually activate transcription factors for induction of ER chaperones, such as GRP78, or phosphorylate eIF2α for inhibition of synthesis of new proteins. These processes are collectively called unfolded protein response (UPR) (Imaizumi et al., 2001). Excessive and prolonged stresses, however, lead cells to apoptosis (Schroder and Kaufman, 2005). ER stress-induced apoptosis is associated with a range of diseases, including ischemia/reperfusion injury, neurodegeneration and diabetes (Xu et al., 2005).

There are two major pathways that induce apoptotic cell death. One is the extrinsic pathway, in which ligation of death receptors by death ligands is followed by recruitment of adaptor molecules and activation of caspase-8 or caspase-10. The other is the intrinsic pathway, in which the release of cytochrome c from mitochondria triggers the formation of the apoptosome composed of Apaf-1, pro-caspase-9, dATP, and cytochrome c. Apoptosome formation results in the activation of executioner caspases including caspase-3, -6, and -7. The precise molecular mechanisms of ER stress-induced apoptosis, however, have not been fully elucidated (Xu et al., 2005). CHOP, a transcription factor induced under ER stress, has been reported to be involved in ER stress-induced apoptosis by reducing the expression of Bcl-2 (McCullough et al., 2001). Indeed, CHOP-deficiency causes resistance to ER stress-induced cell death both in vitro and in vivo (Zinszner et al., 1998). ER stress also leads to increase cytosolic calcium levels, which induces the activation of m-calpain. Activated m-calpain cleaves Bcl-Xl and proteolytically activates caspase-12 (Nakagawa and Yuan, 2000). Processed-caspase-12 reportedly activates caspase-9 independent of Apaf-1, followed by activation of caspase-3 (Rao et al., 2002). Several groups also reported that ER stress increases reactive oxygen species (ROS), and antioxidant protects cells from ER stress-induced cell death (Cullinan and Diehl, 2004; Harding et al., 2003; Haynes et al., 2004; Mauro et al., 2006). The stress kinase pathway is also associated with ER stress-induced apoptosis.
During ER stress, Ask1, one of mitogen-activated protein (MAP) kinase-kinase (MAPKK) kinases, is recruited to oligomerized IRE1 complex containing TRAF2, and is activated for the downstream activation of JNK (Nishitoh et al., 2002). In addition, it is reported that caspase-8 or c-Abl is involved in ER stress-induced apoptosis (Ito et al., 2001; Jimbo et al., 2003). Such complexity may be because the signaling pathways leading to ER stress-mediated apoptosis might vary in different cell types and in different stresses.

Murine caspase-12 is a member of the inflammatory group of the caspase family, and study of the caspase-12-deficient mice revealed that it specifically involves in ER stress-induced cell death (Martinon and Tschopp, 2004; Nakagawa et al., 2000). In humans, the caspase-12 gene has a single nucleotide polymorphism that results in the production of either a truncated or full-length caspase-12 protein (Saleh et al., 2004). However, the full-length human caspase-12 appears to be enzymatically inactive and functions as a dominant-negative regulator of proinflammatory signaling pathways rather than in ER stress-induced cell death pathway. As an alternative to caspase-12 in human, caspase-4 is involved in ER stress-induced cell death pathway (Hitomi et al., 2004a). Both murine caspase-12 and human caspase-4 are localized to the ER and cleaved specifically by ER stress (Hitomi et al., 2004a; Hoppe and Hoppe, 2004; Nakagawa et al., 2000).

In the current study, we investigated the possible involvement of the apoptosome and mitochondria in ER stress-induced cell death pathways using murine embryonic fibroblasts (MEFs) deficient for Apaf-1. Just like Bcl-XL-expressing MEFs, Apaf-1-deficient MEFs showed more resistance to ER stress-inducing reagents as compared with wild type cells. Although caspase-12 activation was observed in wild type, but not in Apaf-1-deficient, MEFs under ER stress, neither overexpression nor knockdown of caspase-12 gene affected susceptibility of the cells to ER stress-induced cell death. In the kidneys of Apaf-1-deficient mice, apoptosis induced by in vivo administration of tunicamycin was remarkably suppressed as compared with that of wild type mice. Our data suggest that ER stress-induced apoptosis is dependent on the mitochondrial pathways of apoptosis involving Apaf-1 and also that caspase-12, activated downstream of Apaf-1, plays little, if any, role in ER stress-induced apoptosis.

**Results**

**Apaf-1**<sup>–/–</sup> MEFs are resistant to ER stress-induced cell death

Apaf-1 is the essential adaptor protein in the intrinsic, mitochondrial pathways of apoptosis and, along with cytochrome c, dATP and caspase-9, forms to the apoptosome (Li et al., 1997; Zou et al., 1997). To investigate the role of Apaf-1 in ER stress-induced cell death, we examined survival of Apaf-1<sup>+/+</sup> or Apaf-1<sup>–/–</sup> primary MEFs against tunicamycin, an inhibitor of N-linked glycosylation, or thapsigargin, an inhibitor of sarcoplasmic-endoplasmic reticulum Ca<sup>2+</sup> ATPase. Both drugs are well characterized as inducers of ER stress and excessive treatments lead to cell death (Kaufman, 1999). First, we checked the growth rate of Apaf-1<sup>+/+</sup> and Apaf-1<sup>–/–</sup> MEFs and found that they proliferated similarly (data not shown). Next, cell survival after stimulation with the ER stress-inducing drugs was examined. Apaf-1<sup>+/+</sup> MEFs died after treatment with these drugs (Fig. 1A). Contrary to the previous report by Rao et al. that Apaf-1-deficient immortalized MEFs, Sak2 cells, died just like wild type NIH/3T3 cells (Rao et al., 2002), Apaf-1<sup>–/–</sup> MEFs showed significant resistance to ER stress-induced cell death over Apaf-1<sup>+/+</sup> MEFs (Fig. 1A). This was checked in more than six different clones of MEFs from more than three different heterozygous intercrosses. This was also the case for MEFs immortalized by SV40 large T antigen (data not shown).

Apaf-1 is required for cleavage of caspase-3 during ER stress-induced cell death

To examine further whether Apaf-1 plays a role of induction of ER stress, we detected caspase-3 activation by western blotting. First, we evaluated induction of GRP78, an indicator of ER stress, by western blotting. Induction of GRP78 by treatment of either tunicamycin or thapsigargin in Apaf-1<sup>+/+</sup> MEFs was comparable to that of Apaf-1<sup>–/–</sup> MEFs (Fig. 1B), confirming that ER stresses were similarly induced both in Apaf-1<sup>+/+</sup> MEFs and Apaf-1<sup>–/–</sup> MEFs. For caspase-3 activation, although treatment with tunicamycin or thapsigargin induced the cleavage of caspase-3 to p17 fragments in Apaf-1<sup>+/+</sup> MEFs at 12-24 hours and thereafter, caspase-3 activation was not observed in Apaf-1<sup>–/–</sup> MEFs under the same condition (Fig. 1B). It was thus demonstrated that ER stress-induced cleavage of caspase-3 is dependent on Apaf-1.

While showing significant resistance to ER stress-induced cell death, Apaf-1<sup>+/+</sup> MEFs gradually died during long culture periods (Fig. 1C). To characterize the cell death induced by ER stress in Apaf-1<sup>–/–</sup> MEFs, we observed the morphological features of cell death using transmission electron microscopy (TEM) (Fig. 1D). Apaf-1<sup>+/+</sup> MEFs showed typical apoptotic changes, such as chromatin condensation, nuclear fragmentation, and plasma membrane blebbing at 48 hours after stimulation. Although Apaf-1<sup>–/–</sup> MEFs showed marginal perinuclear chromatin condensation and cytoplasmic vacuolization, typical apoptotic changes were not observed by 72 hours after stimulation. Nuclear swelling and massive vacuolization observed in Apaf-1<sup>–/–</sup> MEFs rather resembled necrotic cell death.

**Bcl-X<sub>L</sub> protects MEFs from ER stress-induced cell death**

Bcl-X<sub>L</sub> is one of the most potent anti-apoptotic members of the Bcl-2 family proteins. Reportedly, it protects cells from ER stress-induced cell death in various types of cells (Morishima et al., 2004; Obeng and Boise, 2005; Srivastava et al., 1999). Unlike Bcl-2, which may localize ER and affect its function, Bcl-X<sub>L</sub> specifically localizes to mitochondria outer membrane with its C-terminal transmembrane domain (Kaufmann et al., 2003). To examine the effect of Bcl-X<sub>L</sub> expression on ER stress-induced cell death, both Apaf-1<sup>+/+</sup> and Apaf-1<sup>–/–</sup> MEFs were transduced with bel-xl gene by a retrovirus vector. We confirmed that overexpressed-Bcl-X<sub>L</sub> localized preferentially to mitochondria (data not shown). The retrovirus-mediated expression of Bcl-X<sub>L</sub> partially protected Apaf-1<sup>–/–</sup> MEFs from ER stress-induced cell death at approximately the same level with Apaf-1<sup>–/–</sup> MEFs (Fig. 2A). Consistent with this Bcl-X<sub>L</sub>-mediated resistance to ER stress-induced cell death, cleavage of caspase-3 to active form was significantly reduced in MEFs overexpressing Bcl-X<sub>L</sub> against tunicamycin and thapsigargin (Fig. 2B and data not shown). These data, along with the resistance of Apaf-1<sup>–/–</sup> cells against ER stress-induced cell death...
death, clearly indicate that ER stress-induced cell death is dependent on the mitochondrial pathway of apoptosis. However, similar to Apaf-1+/− MEFs, Bcl-XL-overexpressing MEFs showed cell death at later time points (Fig. 2C). Interestingly, Bcl-XL conferred Apaf-1−/− MEFs additional resistance against ER stress-induced cell death (Fig. 2C).

Cytochrome c is released from mitochondria during ER stress-induced cell death

Next, to further substantiate the relationship between ER stress-induced cell death and mitochondria, we investigated the release of cytochrome c from mitochondria under ER stress. At the 12 and 24 hours after stimulation, we observed that cytochrome c was released from mitochondria to cytosol in both Apaf-1+/− and Apaf-1−/− MEFs stimulated with thapsigargin or tunicamycin (Fig. 3 and data not shown). Simultaneously, translocation of BAX, a pro-apoptotic member of the Bcl-2 family, was also detected, indicating the involvement of BAX in ER stress-induced cell death (Martinou and Green, 2001; Ruiz-Vela et al., 2005).
Apaf-1 and ER stress-induced apoptosis

Caspase-12 is downstream of Apaf-1 in ER stress-induced apoptosis and does not affect cell viability in MEFs

Caspase-12 is phylogenetically a member of the inflammatory group of the caspase family, and proteolytically activated under ER stress-induced cell death specifically (Martinon and Tschopp, 2004; Nakagawa et al., 2000). Given the dependence of ER stress-induced cell death on Apaf-1 and mitochondrial pathways demonstrated above, we investigated whether caspase-12 cleavage during ER stress-induced cell death requires Apaf-1. As has been shown previously (Rao et al., 2001), caspase-12 was cleaved into a size of 42 kDa in ER stress-induced cell death in wild type MEFs (Fig. 4A). This cleavage, however, was not observed in Apaf-1–/– MEFs. Furthermore, a caspase-3 specific inhibitor, Ac-DNLD-CHO, inhibited the activation of caspase-12 during ER stress. Taken together, these data indicate that caspase-12 activation is dependent on Apaf-1 and also on caspase-3 activation. As expected, etoposide, a topoisomerase II inhibitor that causes genostress but not ER stress, did not induce caspase-12 activation.

Caspase-12 is downstream of Apaf-1 in ER stress-induced apoptosis

Previous reports showed that caspase-12 is processed at amino acids T132/A133 and K158/T159 by m-Calpain (Nakagawa and Yuan, 2000), at D94 by caspase-7, and at D318 or D341 by autoprocessing of caspase-12 (Fujita et al., 2002; Rao et al., 2001). As caspase-12 was cleaved downstream of Apaf-1/caspase-3 in our experimental condition, we tested whether a mutant form of caspase-12, in which amino acid D94 was substituted with A, was cleaved during ER stress. To this end, we constructed a plasmid expressing caspase-12 with

Fig. 2. Bcl-XL protects MEFs from ER stress-induced cell death. (A) Cell survival of MEFs overexpressing Bcl-XL. bcl-xl was introduced into MEFs with retrovirus-mediated transduction system. Apaf-1+/– (WT) or Apaf-1–/– (KO) MEFs with or without bcl-xl transduction were treated with tunicamycin or thapsigargin for 24 hours. Cell survival was analyzed as described in Fig. 1A. (B) Caspase-3 activation in MEFs overexpressing Bcl-XL. Cells as in A were analyzed for expression of GRP78 and for proteolytic activation of caspase-3. (C) Time course of ER stress-induced cell death of in Bcl-XL-overexpressing MEFs. Cells as in A were analyzed for cell viability after treatment with either 1 μg/ml tunicamycin or 1 μM thapsigargin for indicated hours. Shown are mean ± s.d. Experiments were repeated three times with similar results. Note that the difference of kinetics of cell death in Apaf-1–/– MEFs from Fig. 1C may be due to the infection of retrovirus.

Fig. 3. Cytochrome c is released from mitochondria in MEFs in response to ER stress. Apaf-1+/– MEFs were treated with either thapsigargin or tunicamycin for 24 hours. Cells were fixed and analyzed for intracellular localization of cytochrome c and BAX by immunofluorescence. Arrowheads show cells in which cytochrome c was released from mitochondria and BAX was translocated into mitochondria.
D94A mutation and a FLAG-tag at its N terminus, and introduced into Apaf-1+/– MEFS. While wild type caspase-12, either endogenous or transduced, was cleaved in MEFS in response to ER stress, the mutated caspase-12 was not properly cleaved but gave a faint band with slower gel mobility (see * in Fig. 4B). We did not detect the cleaved band in the mutant caspase-12 sample with antibody against the FLAG tag flanking the cleaved fragment (data not shown). These data suggested that caspase-12 is cleaved at D94 by caspase-3 or downstream of it. The mutant caspase-12 was degraded presumably by calpain or other proteases during ER stress-induced cell death process, resulting in the reduction in the amount of unprocessed form.

Next we investigated if altered expression of caspase-12 would affect cell survival of MEFS during ER stress. First, Apaf-1+/– or Apaf-1–/– MEFS were transduced with wild type casp-12 by retrovirus for overexpression of the gene. Although Kalai et al. reported caspase-12 overexpression induced cell death in HEK293T cells (Kalai et al., 2003), sensitivity to ER stress-induced cell death was not altered at 24 hours after treatment with tunicamycin (Fig. 4C). Furthermore, proteolytic activation of caspase-3 was not induced by the overexpression of caspase-12 in MEFS regardless of Apaf-1 deficiency (Fig. 4D). Next, we conducted a knockdown experiment for caspase-12. Among different three RNAi constructs for targeting caspase-12 different sequences, C12 siRNA-3 clearly reduced the expression of caspase-12 in MEFS (Fig. 4E). However, in these MEFS with reduced expression of caspase-12, the sensitivity to ER stress-induced cell death was not altered (Fig. 4F). These indicate that, in MEFS, caspase-12 is downstream of Apaf-1, cleaved by caspase-3 and possibly caspase-7, but not involved in ER stress-induced cell death.

Apaf-1-deficiency protects cells from ER stress-induced apoptosis in vivo

We finally investigated the impact of Apaf-1-deficiency in an in vivo model. Although most Apaf-1–/– mice die perinatally, some mutant mice normally grow up to adulthood with fertility (Honarpour et al., 2000; Okamoto et al., 2006). It was previously reported that injection of a single dose of tunicamycin induces a characterstic renal lesion in mice, which is mainly caused by apoptosis of the renal tubular
epithelium (Chae et al., 2004; Marciniak et al., 2004; Nakagawa et al., 2000; Zinszner et al., 1998). We tested the sensitivity of age- and sex-matched Apaf-1+/− and Apaf-1−/− mice to tunicamycin. Tunicamycin injection resulted in increase in GRP78 protein level in both Apaf-1+/− and Apaf-1−/− mice (Fig. 5A). Gross inspection of the kidneys of tunicamycin-injected Apaf-1+/− mice revealed conspicuous zonal drop-out of epithelial cells and accumulation of a large number of TUNEL-positive cells (Fig. 5B,C). By contrast, Apaf-1−/− mice exhibited much milder damages and no TUNEL-positive cells in the kidney. These data indicate that Apaf-1 plays an important role in ER stress-induced apoptosis in vivo.

Discussion

Despite the importance and possible involvement of the ER stress in various diseases (Xu et al., 2005), the pathway of ER stress-induced cell death has not been fully elucidated. In the present paper, we examined the role of Apaf-1 and mitochondria in ER stress-induced cell death both in vitro and in vivo. Under our experimental conditions using primary MEFs, Apaf-1−/− MEFs showed no caspase-3 activation and were resistant to ER stress-induced cell death. Furthermore, in response to ER stress, the release of cytochrome c from mitochondria accompanied by the translocation of BAX to mitochondria was observed. Bcl-XL expression protected Apaf-1−/− MEFs from ER stress-induced cell death to similar extent to Apaf-1−/− MEFs. While caspase-12 was activated downstream of Apaf-1 in response to ER stress, it played little, if any, role in the ER stress-induced cell death. Apaf-1-deficiency also protected epithelial cells from ER stress-induced cell death in the kidney in vivo. These data showed the importance of Apaf-1 and the mitochondrial pathway in ER stress-induced cell death. Contrary to this, Rao et al. have reported that Sak-2 cells, Apaf-1-deficient immortalized MEFs, died comparably with NIH/3T3 cells and induced the processing of caspase-3, -7, -9 and -12 under their ER stress conditions (Rao et al., 2002). Although Apaf-1-deficient MEFs died at a later time point, we could not observe the processing of caspase-3 and -12 in our experimental conditions even in MEFs immortalized by the SV40 large T antigen. The discrepancy may be caused by the type of transformation factor, possible involvement of m-calpain activation (Nakagawa and Yuan, 2000), or the difference of the genetic background of MEFs. Phenotypes of knockout mice may occasionally depend on their genetic background. Recently, Di Sano et al. have reported the similar findings that ER stress-induced apoptosis is dependent on Apaf-1 and independent of caspase-12 (Di Sano et al., 2005).

We demonstrated that caspase-12 is cleaved downstream of Apaf-1 at the site of D94 by caspase-3 in MEFs. Although Nakagawa and Yuan previously reported that m-calpain cleaves caspase-12 under ER stress (Nakagawa and Yuan, 2000), we did not detect activity of m-calpain using fluorescent substrates (data not shown). These are consistent with the data observed in in vitro assay using recombinant caspase-12 and -3 (Hoppe and Hoppe, 2004). Furthermore, knockdown or overexpression experiments showed that caspase-12 plays little role in ER stress-induced apoptosis of MEFs. Recent papers by Obeng et al. and by Di Sano et al. also demonstrated similar findings that ER stress-induced cell death is independent of caspase-12 but depends on the apoptosome (Di Sano et al., 2005; Obeng and Boise, 2005). Caspase-12 is,
however, localized on the cytoplasmic side of the ER membrane (Nakagawa et al., 2000; Rao et al., 2001), and is actually cleaved in our ER stress condition, too. These indicate the possible involvement of caspase-12 in ER stress. Caspase-12 is phylogenetically one of the inflammatory group caspases (Martinon and Tschopp, 2004). It has been reported that murine caspase-12 expression is induced by IFN-γ (Kalai et al., 2003) and the expected NF-κB- and AP1-binding sites are present in its promoter region (Oubrahim et al., 2005). In humans, a caspase-12 isozyme functions as dominant-negative regulator of pro-inflammatory signaling pathway (Saleh et al., 2004). In addition, ER stress also induces NF-κB activation mediated by TRAF2 (Mauro et al., 2006). These data indicate a role of caspase-12 in inflammation but not apoptosis. Furthermore, Saleh et al. have recently reported that murine caspase-12 also functions the regulator of inflammation in vivo (Saleh et al., 2006). It is possible that ER stress activates some of the pro-inflammatory signal transduction pathway associated with the innate immunity in situations such as viral infection.

Although showing resistance to ER stress-induced cell death, Apaf-1−/− MEFs also gradually died. The cells showed non-apoptotic features, such as many vacuoles. Non-apoptotic, necrosis-like cell death of Apaf-1−/− cells at later stages of apoptotic stimulation was also reported previously (Chautan et al., 1999; Miyazaki et al., 2001). These vacuoles might be ERs, because ER stress is known to induce swelling of the ER lumen and dissociation of ribosomes from rough ER (Hitomi et al., 2004b). In some neurodegenerative diseases where ER stress has been allegedly involved, morphological changes including formation of cytoplasmic vacuoles have been reported while accumulation of abnormal proteins and cell death play significant roles in the development of the diseases (Kobayashi et al., 2002; Mizuno et al., 2003). In this view, the cell death with vacuoles observed in Apaf-1−/− MEFs may have physiological relevance.

Bcl-XL conferred resistance to wild type cells against ER stress-induced cell death just like Apaf-1-deficient cells, confirming the involvement of Apaf-1/mitochondrial pathways of apoptosis in ER stress-induced cell death. Interestingly, Bcl-XL bestowed additional resistance to Apaf-1−/− MEFs against ER stress-induced cell death. Lines of evidence have shown that various pro-apoptotic factors are released from mitochondria including Smac/DIABLO (Du et al., 2000), Omi/HtrA2 (Suzuki et al., 2001), AIF (Joza et al., 2001; Susin et al., 1999), and Endo G (Li et al., 2001) as well as cytochrome c. Smac/DIABLO and Omi/HtrA2 block IAP-family proteins, which inhibit caspases at post-mitochondrial phases. AIF and Endo G cause large fragmentation of chromosomal DNA leading cell death either dependent on or independent of caspases (Aarnout et al., 2003). Considering the lack of caspase-3 activation in Apaf-1−/− MEFs, the additional effects of Bcl-XL may be due to blocking the release of AIF and Endo G from mitochondria by mitochondrial membrane stabilization. Another possibility is that Bcl-XL may function at the ER membrane. Bcl-XL blocks the release of pro-apoptotic factors by blocking functions of Bax and Bak channels on mitochondria. Therefore, Bcl-XL may regulate the function of Bax and Bak at the ER (Zong et al., 2003). However, neither Bcl-XL overexpression nor Apaf-1-deficiency protected cells from ER stress-induced cell death observed at later phases of stimulation. Consistent with the in vitro finding, in vivo administration of tunicamycin still induced epithelial cell death in Apaf-1−/− mice, albeit reduced in degree. These data thus indicate that the ER stress induces Apaf-1-independent, non-apoptotic cell death, as well as Apaf-1/mitochondria-dependent apoptosis. The non-apoptotic cell death observed in the absence of Apaf-1 (Chautan et al., 1999; Miyazaki et al., 2001) may be physiologically veiled by Apaf-1- and caspase-3-dependent canonical apoptosis, which occurs faster. The presence of non-apoptotic cell death induced by genostresses and ER stresses indicates that targeting of apoptotic pathway may not be enough to suppress cell death in diseases such as neurodegenerative disorders.

In conclusion, in the present study we provide evidence that Apaf-1 and mitochondria are critically involved in ER stress-induced cell death in MEFs as well as in renal epithelial cells. Further elucidation of the molecular mechanisms linking ER stress to mitochondrial damages will no doubt shed light to the understandings of and therapeutic intervention of various diseases in which ER stress play pathological roles.

Materials and Methods

Cell culture and reagents

Apaf-1−/− or Apaf-1+/− mouse embryonic fibroblasts were obtained from E14.5 embryos born to heterozygous intercrosses and cultured in DMEM supplemented with 10% FCS, L-glutamine and β-mercaptoethanol. All experiments were done with cells at between three and five population doublings level (PDL). Tunicamycin, thapsigargin and etoposide were purchased from Sigma. The Caspase-3-specific inhibitor Ac-DNLD-CHO was purchased from the Pepitide Institute.

Plasmids and constructs

A human bcl-xl cDNA was subcloned into the retroviral expression vector, pMX-IRES-PURO (Nosaka et al., 1999). Murine casp-12 cDNA obtained from MEFs was cloned into pFLAG-CMV2 (Sigma). The substitution mutant casp-12 D94A cDNA was generated using standard polymerase chain reaction method. Wild type and mutant cDNAs tagged with FLAG-epitope were subcloned into pMX-IRES-PURO.

Detection of cell death

MEFs were plated in a 24-well dish at 5×10^4 cells per well the day prior to treatment. The cells were left untreated or treated with tunicamycin or thapsigargin at indicated concentrations. At various time points after treatment, cells were trypsinized and collected with the supernatants, and lysed with a mixture of 5 mM Tris, 150 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail. Cell lysates were then resolved on 12% SDS-PAGE gels and transferred onto nitrocellulose membranes, and incubated with antibodies reactive to G1 (Santa Cruz), Bcl-XL, or FLAG-epitope, respectively. The membranes were developed with western blotting solution containing 100 mM sodium pyrophosphate, 500 mM β-glycerophosphate, 1 mM sodium vanadate, and 1% non-fat dry milk. The membranes were then washed in TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) and incubated with a secondary antibody conjugated to horseradish peroxidase diluted in TBS-T. The membranes were washed and developed with chemiluminescent substrate reagent.

Electron microscopy

Before or after apoptotic treatment, MEFs were fixed on ice with 1× fixing buffer (2.5% glutaraldehyde, 0.1 M sucrose, and 3 mM CaCl2 in 0.1 M cacodylate buffer [pH 7.4]). The cells were fixed in 1% OsO4 for 1 hour at 4°C, dehydrated in graded ethanol and in propylene oxide, and embedded in Epon 812 resin. Thin sections were stained with 5% uranyl acetate for 30 minutes and lead acetate for 25 minutes. The sections were then examined under a JEM-2000-EX (JEOL, Tokyo) electron microscope.

Immunocytochemistry for cytochrome c

MEFs were grown on 1 mm glass coverslips for 24 hours and stimulated with tunicamycin (1 μg/ml) or thapsigargin (0.1 μM). Twenty-four hours after stimulation, cells were fixed with 4% paraformaldehyde, and then permeabilized with 0.1% Triton X-100 in phosphate buffered saline at room temperature for 5 minutes. MEFs were immunostained with the antibody reactive to cytochrome c (BD Pharmingen) and BAX (Upstate).

Retrovirus-mediated gene transduction

Human bcl-xl, murine casp-12, FLAG-casp-12, or FLAG-casp-12(D94A) subcloned into pMX-IRES-PURO were transfected into the PLAT-E packaging cell line, and cotransfected with the retroviral vectors. The virus-containing supernatant was harvested, concentrated using a centrifuge column (Amicon), and then added to MEFs. After 48 hours, MEFs were stained with the antibody reactive to cytochrome c (BD Pharmingen) and BAX (Upstate).
line using the calcium phosphate method to obtain viruses. MEFs were infected with the virus, treated with polybrene (Sigma) a day after infection, and then culture medium was replaced with the selection medium containing paromycin. Twenty-four hours after selection, surviving cells were used for experiments.

Knockdown of caspase-12

The mammalian expression vector, pSUPER.retro.puro (Oligoengine) was used for expression of siRNA targeting murine caspase-12 in MEFs. Sense and anti-sense oligonucleotides, corresponding to (5′-3′) 522-542 (C12 si1), 535-555 (C12 si-2) and 1075-1095 (C12 si-3) of murine casp-12 cDNA (GenBank accession no. NM_009080) were annealed and subcloned into pSUPER.retro.puro vector, respectively.

In vivo analysis of ER stress-stress-induced cell death

Age- and sex-matched Apaf-1<sup>−/−</sup> and Apaf-1<sup>+/+</sup> mice (6- to 8-week-old) were given a single injection of tunicamycin (3 μg/gm body weight, intraperitoneally (i.p.)). After 2 or 3 days, mice were sacrificed, and their kidneys were removed. One kidney was lysed by NP40 lysis buffer for western blotting analysis, and the other was fixed in 4% paraformaldehyde for hematoxylin and eosin staining and deoxyribonucleic acid transferase-mediated dUTP nick-end labeling (TUNEL) assay and 4′,6-diamidino-2-phenylindole (DAPI) staining. Fixed samples were paraffin-embedded and 5 μm sections were mounted on glass slides. TUNEL assay was performed using ApopTag In Situ Apoptosis detection kit (CHEMICON) according to the manufacturer’s instructions.

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