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Caspase-Independent Cell Death and Mitochondrial Disruptions Observed in the Apaf1-Deficient Cells[1]

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Apaf1 is a critical molecule in the mitochondria-dependent apoptotic pathway. Here we show that Apaf1-deficient embryonic fibroblasts died at a later phase of apoptotic induction, although these cells were resistant to various apoptotic stimulants at an early phase. Neither caspase 3 activation nor nuclear condensation was observed during this cell death of Apaf1-deficient cells. Electron microscopic examination revealed that death in response to apoptotic stimulation resembled necrosis in that nuclei were round and swollen with low electron density. Necrosis-like cell death was also observed in wild-type cells treated with z-VAD-fmk. Mitochondria were not only morphologically abnormal but functionally affected, since mitochondrial transmembrane potential ($\Delta\Psi m$) was lost even in cells with intact plasma membrane integrity. These mitochondrial alterations were also observed in the wild-type cells dying of apoptosis. Combined, these data suggest that cells without caspase activation, such as Apaf1-deficient cells or cells treated with caspase inhibitors, die of necrosis-like cell death with mitochondrial damage in response to "apoptotic stimulation."

Key words: Apaf1, apoptosis, electron microscopy, mitochondria, necrosis.

Apoptosis is a process of cell death that was originally described by its morphological characteristics including cell shrinkage and chromatin condensation (*1*). Apoptosis is essential for normal development and homeostasis in multicellular organisms and also serves as a defense mechanism to eliminate harmful cells, such as tumor cells and cells infected by viruses (*2*). A recent advance has shown that mitochondria play essential roles in apoptosis (*3–6*). While mitochondria produce metabolic energy in the form of ATP, they also contain and release proteins that are involved in the apoptotic cascade, such as cytochrome *c* (Cyto *c*) and some caspases (*7*). Cyto *c*, an essential component of the respiratory chain of the mitochondria, is released in response to various apoptotic stimuli (*8, 9*) and binds the apoptotic protease activating factor 1 (Apaf1), leading to the formation of apoptosome. Apoptosome then proteolytically activates caspase (Casp) 9, and the activated Casp9 cleaves the downstream caspases including Casp3, 6, and 7, bringing about apoptotic cell death by digesting essential cellular proteins (*10, 11*). Thus, deficiency of the essential component(s) of the mitochondrial apoptotic pathways renders the cells remarkably resistant to apoptotic stimulation, as shown in gene-disrupted mice (*12–17*).

Although apoptosis has been shown to be beneficial for the clearance of cells, recent evidence shows that a caspase-independent mechanism for commitment to death also exists. For instance, certain apoptotic stimuli or proapoptotic proteins such as Bax induce cell death even in the presence of caspase inhibitors (*18*). To investigate the mechanism of caspase-independent cell death, we employed Apaf1-deficient cells, which show remarkable resistance to apoptotis and marked reduction in caspase activation (*17*)

In this study, we show that apoptosis-resistant Apaf1-deficient cells die of necrosis-like cell death after a longer time of apoptotic stimulation than wild-type cells. We also show that mitochondria are disrupted, both functionally and morphologically, in response to the apoptotic stimuli. Our findings indicate that apoptotic cell death and mitochondrial damage-induced metabolic disturbances occur simultaneously.

MATERIALS AND METHODS

Cell Culture—Wild-type (*Apaf1*$^{+/+}$) or Apaf1-deficient (*Apaf1*$^{-/-}$) mouse primary embryonic fibroblasts (PEFs) were obtained from E14.5 embryos born to *Apaf1* heterozygous intercrosses and grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 7.5% heat-inactivated fetal bovine serum, β-mercaptoethanol, and antibiotics. Early passaged cells were used throughout the experiments.

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Abbreviations: Apafl, apoptotic protease activating factor-1; Casp, caspase; Cyto *c*, cytochrome *c*; DAPI, 4′,6-diamidine-2′-phenylindole dihydrochloride; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; DMEM, Dulbecco's Modified Eagle Medium; $\Delta\Psi m$, mitochondrial transmembrane potential; PEF, primary embryonic fibroblast; PI, propidium iodide; ROS, reactive oxygen species; TEM, transmission electron microscopy.

Induction of Apoptosis and Determination of Cell Death—For induction of apoptosis, 1 × 10^5 wild-type or Apaf1-deficient PEFs were plated in each well of a 24-well dish, then left untreated or treated with staurosporine (2 μM, Sigma), cisplatinum (100 μM, Sigma), or UV irradiation (24 mJ/cm², Spectrolinker XL-1500 UV, Spectronics) as an apoptotic stimulant. In some cases, z-Val-Ala-Asp-fluoromethylketon (z-VAD-fmk, Kamiya Biomedical; 50 μM) or z-Asp-Glu-Val-Asp-fluoromethylketon (z-DEVD-fmk, Kamiya Biomedical; 100 μM) was added as a caspase inhibitor 1 h before apoptotic stimulation. After incubation for various periods, cells were collected by trypsinization along with floating cells and then examined for viability. Determination of cell viability was based on the exclusion of propidium iodide (PI) and a negative reaction for Annexin V binding using a Mebcyto apoptosis kit (MBL, Nagoya). Cells negative for PI uptake and Annexin V binding by flow cytometric analysis were determined as viable.

Caspase Activity Assay—Casp3-like activity (DEVDase activity) in PEFs was measured with a CPP32/Caspase-3 colorimetric protease assay kit (MBL) according to the manufacturer's instructions.

Fluorescence Microscopy Analyses—For nuclear staining, cells were fixed with methanol and stained with 0.5 μg/ml 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI, Boehringer Mannheim) at 37°C for 15 min. Alternatively, for simultaneous detection of nuclear morphological changes, plasma membrane integrity, and mitochondrial membrane potential, cells treated with an apoptotic stimulant were stained with Hoechst 33342 (1 μM; Calbiochem) at 37°C for 15 min, propidium iodide (PI, 50 μg/ml; Sigma) at room temperature for 15 min, and 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] (100 nM; Molecular Probes) at 37°C for 15 min. Cells were examined for apoptotic changes with a fluorescence microscope.

Electron Microscopy—Before or after apoptotic treatment, PEFs were fixed on ice with 1× fixing buffer (2.5% glutaraldehyde, 0.1 M sucrose, and 3 mM $CaCl_2$ in 0.1 M cacodylate buffer [pH 7.4]). The cell were processed through 1% OsO_4 for 1 h 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 min and lead acetate for 25 min, then examined under a JEM-2000-EX (JEOL, Tokyo) electron microscope.

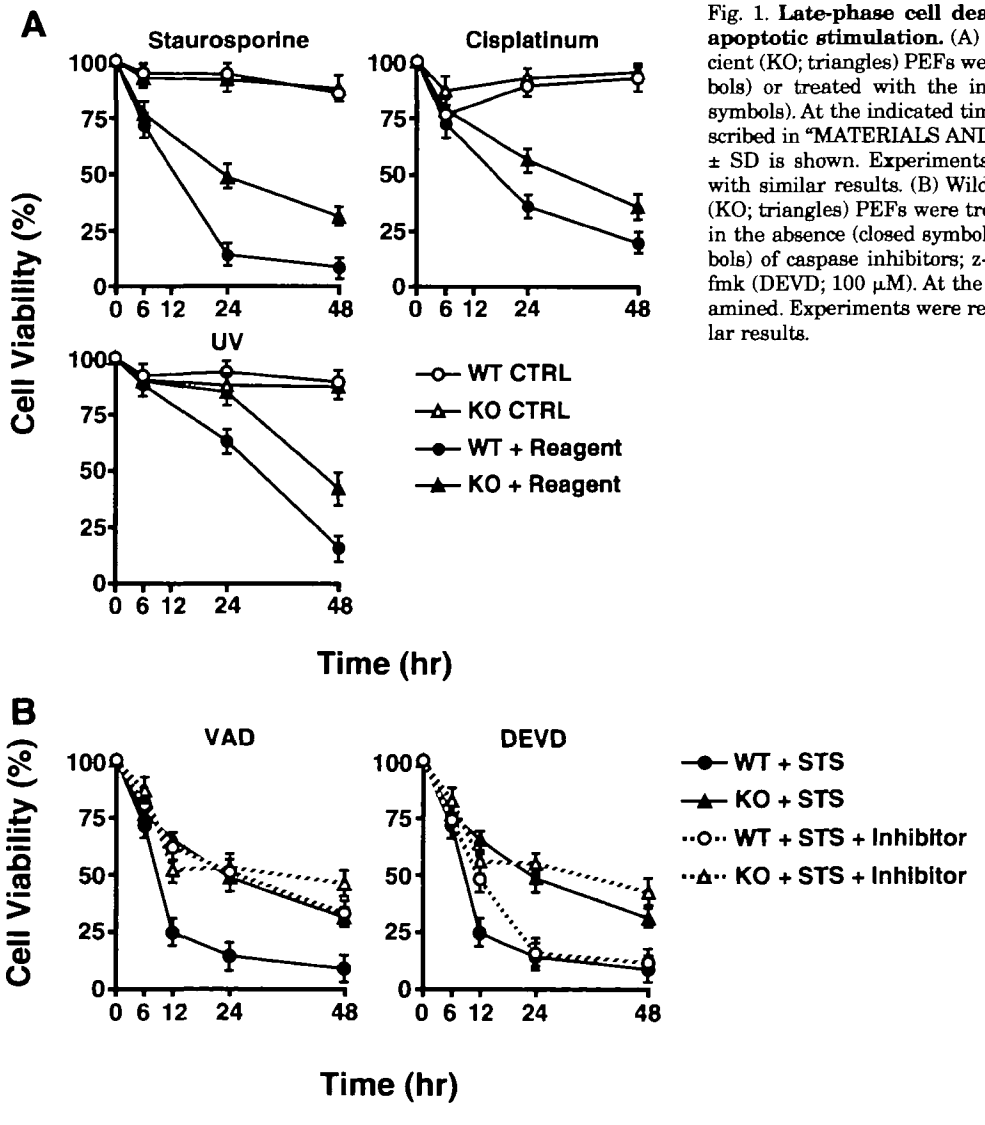

Fig. 1. **Late-phase cell death in Apaf1-deficient PEFs after apoptotic stimulation.** (A) Wild-type (WT; circles) or Apaf1-deficient (KO; triangles) PEFs were either untreated (CTRL; open symbols) or treated with the indicated stimulant (+Reagent; closed symbols). At the indicated times, cell viability was examined as described in "MATERIALS AND METHODS." Mean percent viability ± SD is shown. Experiments were repeated at least three times with similar results. (B) Wild-type (WT; circles) or Apaf1-deficient (KO; triangles) PEFs were treated with staurosporine (STS) either in the absence (closed symbols) or presence (+ Inhibitor open symbols) of caspase inhibitors; z-VAD-fmk (VAD; 50 μM) or z-DEVD-fmk (DEVD; 100 μM). At the indicated times, cell viability was examined. Experiments were repeated at least three times with similar results.

RESULTS

Late-Phase Cell Death of Apaf1-Deficient Cells in Response to Apoptotic Stimulation—The previous report clearly showed that Apaf1-deficient PEFs were resistant to various apoptotic stimulants including anti-cancer drugs (*18*). As shown in Fig. 1A, Apaf1-deficient PEFs were more resistant to various apoptotic stimulants including staurosporine, cisplatinum, and UV irradiation than wild-type PEFs. However, cell viability was gradually lost with all stimulants tested in the later phase of observation: for example, at 48 h after stimulation with staurosporine, the viability of Apaf1-deficient PEFs was only ~30%, while that of wild-type PEFs was ~10%.

To exclude the possibility that these apoptotic stimulants activated the caspase cascade(s) independently of Apaf1 and led to cell death in Apaf1-deficient cells, effects of caspase inhibitors were examined. As shown in Fig. 1B, pretreatment of the Apaf1-deficient PEFs with z-VAD-fmk (a pan-caspase inhibitor) or z-DEVD-fmk (a Casp3 inhibitor) only slightly prevented the cell death of the Apaf1-deficient PEFs; nonetheless, cell viability was gradually lost at 24 to 48 h in response to the apoptotic stimulation. z-VAD-fmk was effective in preventing apoptosis in the wild-type PEFs at 6 to 12 h after staurosporin treatment (Fig. 1B). z-DEVD-fmk were also effective in the wild-type PEFs up to 12 h after staurosporin treatment. However, even in the presence of the caspase inhibitor, the wild-type PEFs also showed cell death in the later phase (24 to 48 h).

Lack of Caspase 3 Activation and Nuclear Condensation in the Apaf1-Deficient Cells—To further investigate the cell death observed in the Apaf1-deficient PEFs at the later phase, we examined the Casp3 activity in the Apaf1-deficient and the wild-type PEFs after apoptotic stimulation. As shown in Fig. 2, activation of Casp3 was observed in the wild-type PEFs after staurosporine, cisplatinum and etoposide (not shown) treatment. However, in the Apaf1-deficient PEFs, no induction of Casp3 activity was detected at 12 and 24 h after the apoptotic stimulation, when some of Apaf1-deficient PEFs were dead (Fig. 1A). Addition of z-VAD-fmk at 50 μM completely inhibited the Casp3 activity in the wild-type PEFs. This indicated that the cell death in Apaf1-deficient PEFs was Casp3-independent, and also independent of caspase activation that was inhibited by z-VAD-fmk.

We also examined the morphological changes in the Apaf1-deficient PEFs after apoptotic stimulation. As depicted in Fig. 3, wild-type PEFs showed nuclear condensation, a typical sign of apoptosis, with brighter DAPI staining at 12 h after staurosporine, cisplatinum, or etoposide (not shown) treatment. In contrast, Apaf1-deficient PEFs showed lower intensity of DAPI staining and no nuclear condensation. Addition of the caspase inhibitor z-VAD-fmk completely inhibited the nuclear condensation in the wild-type PEFs, whereas it had no effect in the Apaf1-deficient PEFs. These data together indicate that the cell death observed in the Apaf1-deficient PEFs after apoptotic stimulation was neither caspase-dependent nor apoptosis.

Loss of Mitochondria Membrane Potential in Cells with Intact Plasma Membrane Integrity—The apoptotic stimulants utilized here are known to trigger the release by mitochondria of caspase-activating proteins upstream of caspase activation. We therefore examined whether mitochondria were functionally affected by the apoptotic stimulants in Apaf1-deficient PEFs by evaluating changes in the mitochondrial transmembrane potential ($\Delta\Psi m$) using the potential-sensitive dye $DiOC_6(3)$ (Fig. 4). Before apoptotic stimulation, both wild-type and Apaf1-deficient PEFs showed intact nuclear morphology by Hoechst 33342 staining, intact $\Delta\Psi m$ by $DiOC_6(3)$ staining, and intact plasma membrane integrity by PI exclusion. As early as 6 h after staurosporine treatment, however, some wild-type and Apaf1-deficient cells showed loss of $\Delta\Psi m$. Most of the cells (both wild-type and Apaf1-deficient) with low $\Delta\Psi m$ signal showed no evidence of PI uptake, indicating the intact plasma membrane integrity. Flow cytometric analysis showed loss of $\Delta\Psi m$ in 12–15 and 6–9% of PI uptake-negative wild-type and Apaf1-deficient PEFs, respectively (not shown). At 12 h after stimulation, some cells (both wild-type and Apaf1-deficient) that had lost $\Delta\Psi m$ were positive for PI uptake (not shown). These data demonstrated that the loss of $\Delta\Psi m$ occurred before cells were shown to be dead by inclusion of PI in both the wild-type and Apaf1-deficient PEFs.

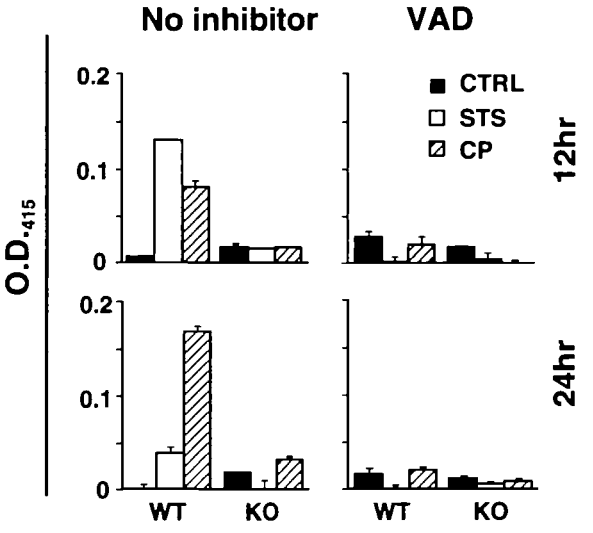

Fig. 2. **Lack of caspase 3 activation in Apaf1-deficient cells in response to apoptotic stimuli.** Wild-type (WT) or Apaf1-deficient (KO) PEFs were either untreated (CTRL, closed columns) or treated with staurosporine (STS, 2 μM; open columns) or cisplatinum (CP, 100 μM; hatched columns) for 12 and 24 h. The caspase inhibitor z-VAD-fmk (VAD) was added at 50 μM. DEVDase activity was measured as absorbance ($O.D._{415}$) based on the cleavage of the substrate p-nitroaniline-DEVD. Mean OD ± standard deviation is shown. Experiments were repeated twice with similar results.

Electron Microscopic Analyses of the Cells—To further investigate the cell death observed in the Apaf1-deficient PEFs after apoptotic stimulation, we examined the ultrastructures of the cells by transmission electron microscopy (TEM) (Fig. 5). TEM analysis of wild-type PEFs showed typical chromatin condensation and nuclear fragmentation, plasma membrane blebbing, and apoptotic body formation at 12 (not shown), 24 and 48 h after stimulation. In contrast, Apaf1-deficient PEFs showed no nuclear fragmentation or condensation up to 48 h, although some cells showed marginal peripheral nuclear condensation. In some Apaf1-deficient PEFs, nuclei appeared large and round

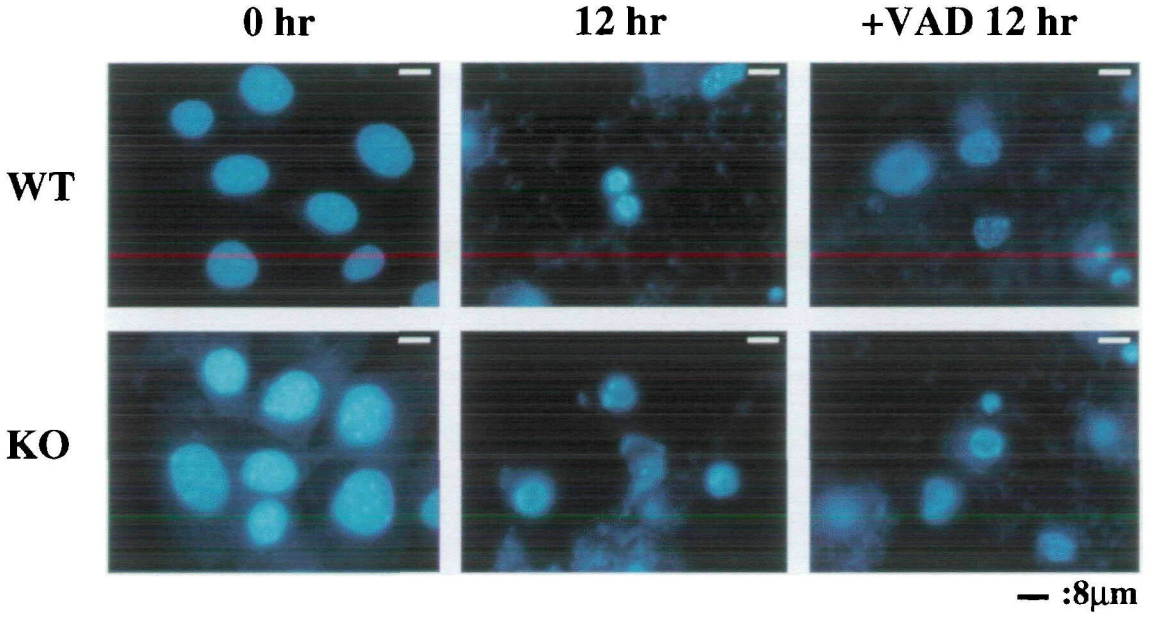

Fig. 3. **Lack of nuclear condensation in Apaf1-deficient cells in response to apoptotic stimulation.** Wild-type (WT) or Apaf1-deficient (KO) PEFs were treated with staurosporine (2 μM) for 0 or 12 h. The caspase inhibitor z-VAD-fmk (VAD) was added at 50 μM. Nuclear staining by DAPI is shown. Scale bsrs = 8 μm.

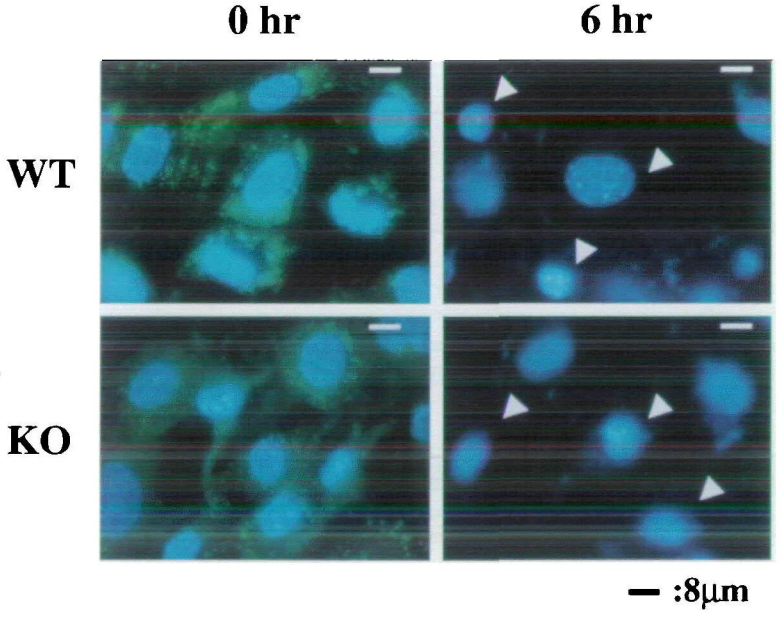

Fig. 4. **Dissipation of mitochondrial transmembrane potential after apoptotic stimulation.** Wild-type (WT) or Apaf1-deficient (KO) PEFs were treated with staurosporine (2 μM) for 0 and 6 h. Cells were stained with Hoechst 33342 (blue), PI (red), and $DiOC_6(3)$ (green), for nuclear morphology, plasma membrane integrity, and mitochondrial transmembrane potential ($\Delta\Psi m$), respectively, as described in materials and methods. Arrowheads; cells with low $\Delta\Psi m$ but negative for PI uptake. Scale bsrs = 8 μm.

with low electron density at 48 h after stimulation. This appearance of nuclei in the Apaf1-deficient PEFs was strikingly similar to that observed in cells that were treated with a high dose of H_2O_2 (5 mM) to induce necrosis. These necrosis-like nuclear changes were also observed when the wild-type PEFs were treated with z-VAD-fmk before the induction of apoptosis.

We then closely examined the mitochondria after apoptotic stimulation. Before apoptotic stimulation, mitochondria in the wild-type and Apaf1-deficient PEFs had an orthodox configuration (Fig. 6). At 6 h after stimulation, mitochondria appeared swollen with a pale matrix and some showed annihilation of cristae structure; the remaining cristae also appeared shredded in the Apaf1-deficient PEFs. This destruction of mitochondrial structure was similarly seen in the wild-type PEFs after apoptotic stimulation. Addition of the caspase inhibitor to both types of cells, which effectively prevented the nuclear morphological changes (Figs. 3 and 5), did not inhibit the swelling of the mitochondria in either the wild-type or the Apaf1-deficient PEFs. In cells treated with H_2O_2 to induce necrosis, the swelling and destruction of mitochondria were also observed.

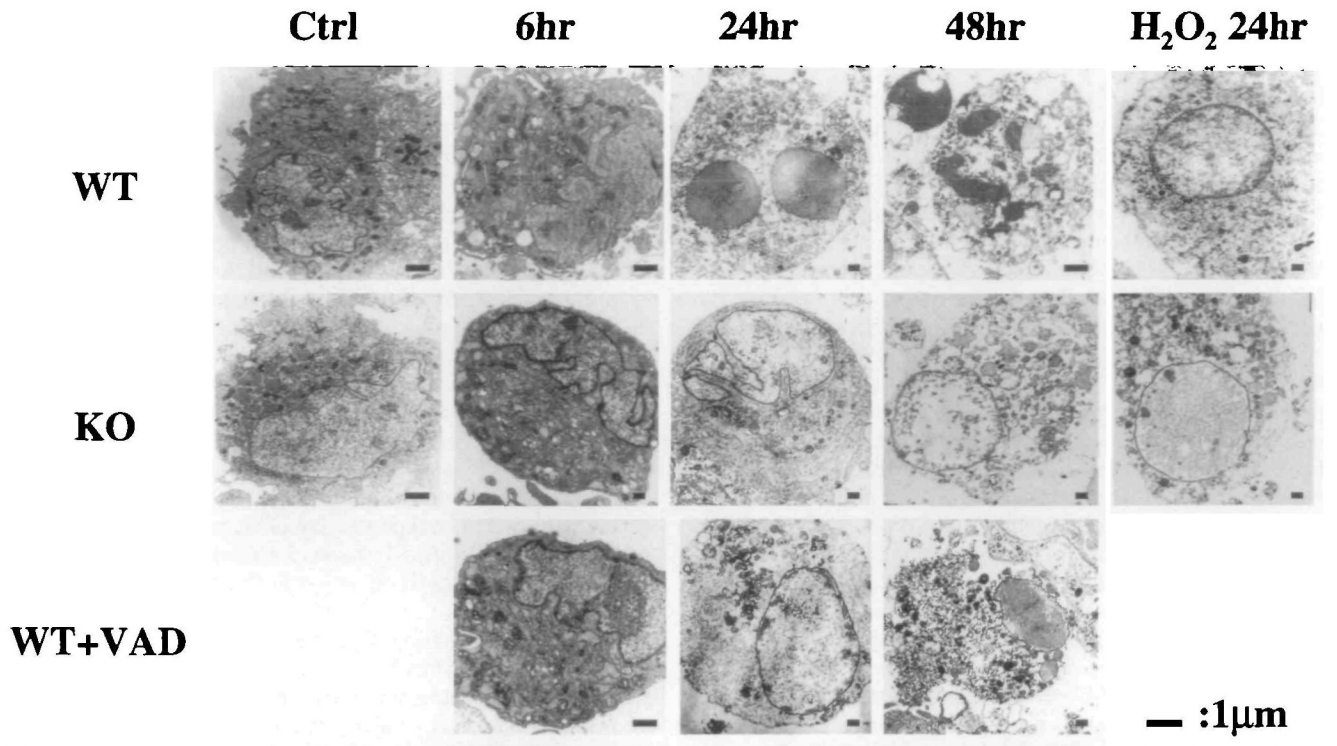

Fig. 5. **Transmission electron microscopic analysis of wild-type and Apaf1-deficient cells.** Wild-type (WT) or Apaf1-deficient (KO) PEFs were treated with staurosporine (2 μM) for 0, 6, 24, or 48 h. The caspase inhibitor z-VAD-fmk was added at 50 μM (WT+VAD). For induction of necrosis, both wild-type and Apaf1-deficient cells were treated with H_2O_2 (5 mM). Note that typical nuclear condensation in the wild-type cells and also nuclear swelling in the Apaf1-deficient cells and wild-type cells treated with z-VAD-fmk is similar to that in the nuclei of H_2O_2-treated cells. Scale bsrs = 1 μm.

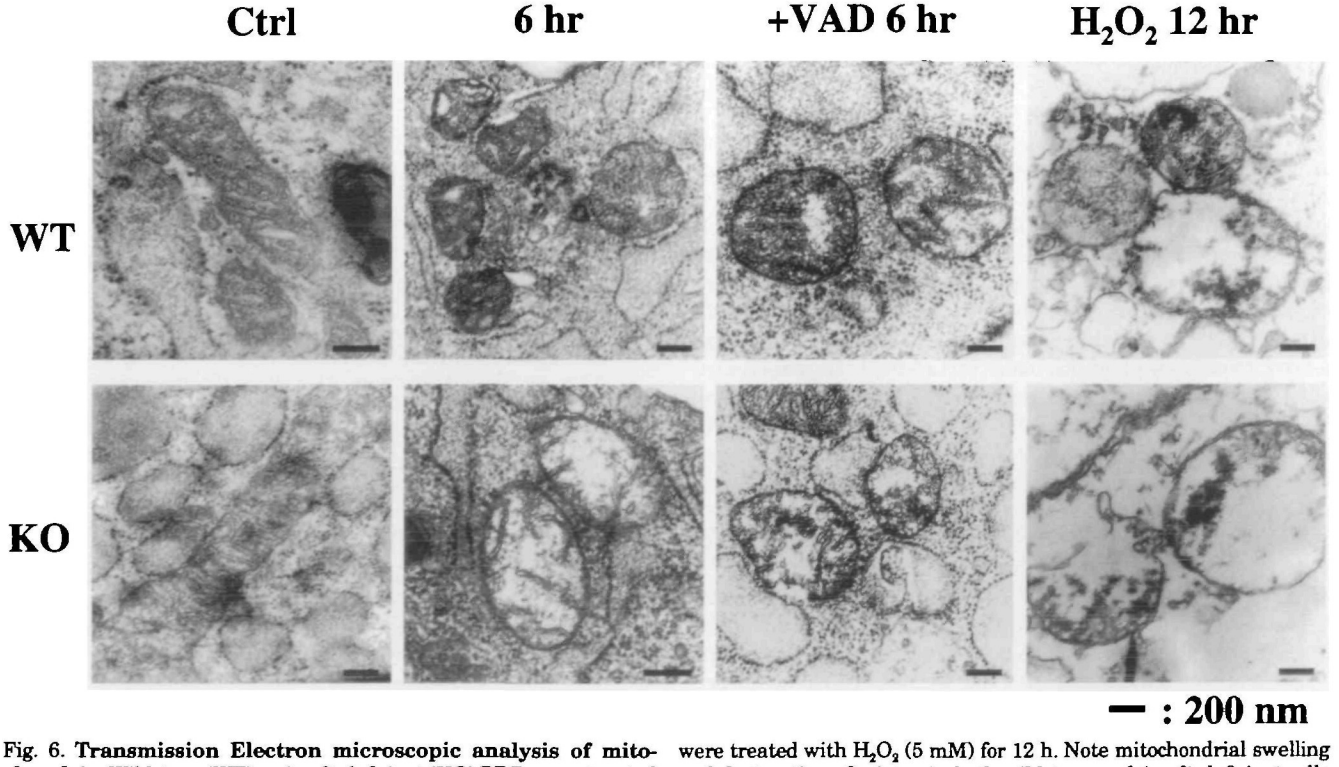

Fig. 6. **Transmission Electron microscopic analysis of mitochondria.** Wild-type (WT) or Apaf1-deficient (KO) PEFs were treated with staurosporine (2 μM) for 0 or 6 h either in the absence or presence (+VAD) of z-VAD-fmk (50 μM). For induction of necrosis, cells were treated with H_2O_2 (5 mM) for 12 h. Note mitochondrial swelling and destruction of cristae in both wild-type and Apaf1-deficient cells even in the presence of the caspase inhibitor. Necrotic cells also showed similar destruction of mitochondria. Scale bars = 200 μm.

DISCUSSION

We have found that Apaf1-deficient PEFs, which had been shown to be resistant to various apoptotic stimuli at an early phase after stimulation, died at a later phase and that this cell death was morphologically similar to necrosis and was caspase-independent. Necrosis-like cell death in Apaf1-deficient embryonic stem cells was also reported by Haraguchi *et al.* (*19*). Until recently, apoptosis and necrosis have been regarded as distinct entities of cell death: while apoptosis is an active energy-dependent process, necrosis is a passive, accidental death that occurs when cells are exposed to an excess of stimulant. Often the intensity of the apoptotic simulation decides whether the fate of cells will be apoptosis or necrosis (*20, 21*). However, these two types of cell death occur simultaneously in tissues (*22, 23*). In the present study, we demonstrated that the same stimulants that induced apoptosis in the wild-type PEFs induced necrosis-like cell death in the Apaf1-deficient PEFs. Our findings suggest that the same stimulation may induce apoptosis or necrosis depending on the involvement of caspases [or related molecule(s) in the apoptotic cascade, such as Apaf1], indicating that some early events are common to both types of cell death, and that downstream events direct cells towards apoptosis or necrosis.

Mitochondrial alterations have been shown to play critical roles in both apoptosis and also in necrosis (*6*). Mitochondrial permeability transition (PT) and a correlating event, disruption of $\Delta\Psi$m, appear to be critical events during the mitochondrial alteration (*5*). Of note, the disruption of $\Delta\Psi$m was observed both in the wild-type and Apaf1-deficient PEFs, indicating that the disruption *per se* is caspase-independent, although caspase-dependent mitochondrial damage was also reported (*24*). Moreover, Fig. 4 demonstrates that the disruption was observed as early as 6 h after apoptotic stimulation in cells with intact plasma membrane integrity and no signs of nuclear condensation (in wild-type PEFs), indicating that the disruption is an early event. Our previous report showed Cyto *c*, a mitochondrial protein, was released in both the wild-type and Apaf1-deficient PEFs at 4 h after apoptotic stimulation (*17*), demonstrating that Apaf1-independent mitochondrial damage occurred at an early phases. Mitochondria were damaged not only functionally but also structurally (Fig. 6). Mitochondria from wild-type and Apaf1-deficient PEFs appeared swollen, and cristae were structurally damaged at 6 h after stimulation. These data together suggest that the mitochondrial damage may be an early event common to apoptosis and necrosis. Once damaged, mitochondria not only induce apoptosis by releasing caspase-activating proteins, but lead cells to necrotic death by disruption of electron transport and energy metabolism and generation of reactive oxygen species (ROS) (*6*).

What are the inducers of mitochondrial alterations in response to apoptotic stimulation? Since mitochondrial damage was observed in both wild-type and Apaf1-deficient PEFs or in cells with no DEVDase activity in the presence of a caspase inhibitor (Figs. 2, 4, and 6), the induction of mitochondrial damage should occur upstream of caspase activation. In response to various apoptotic stimuli, p53 is activated to induce expression of some of apoptosis-related molecules (for review, see Ref. *25*), such as BAX, NOXA, and p53AIP1, which have been shown to induce dissipation of $\Delta\Psi$m, and as such, p53 and p53-induced molecules are most likely to be the inducers of mitochondrial damage observed in this study. ROS, generated in response to p53 activation or as a result of electron transfer failure, is also likely to induce mitochondrial damage. Actually, Pan *et al.* have reported similar mitochondrial swelling and destruction of mitochondrial structures in cells treated with an anti-cancer drug, which was inhibited by radical scavengers (*26*). An alternative, but not mutually exclusive, possibility is the involvement of lysosomal enzymes released from destabilized lysosomes. It is known that if apoptotic mammalian cells do not undergo phagocytosis, they undergo lysosomal destabilization (*27*). Early lysosomal destabilization is not affected by expression of Bcl-2 (*28*), indicating that the destabilization is independent of Apaf1 and caspases. Thus, it is possible that the release of proteases and endonucleases from lysosomes due to the destabilization causes cell death independently of caspase activation. The roles of p53, ROS and lysosomal enzymes in the caspase-independent cell death observed in this study is now under examination.

In summary, we have demonstrated that in the absence of caspase (DEVDase) activation, as in Apaf1-deficient PEFs or PEFs pretreated with a caspase inhibitor, cells die of necrosis-like cell death with dissipation of $\Delta\Psi$m and ultrastructural changes of mitochondria in response to "apoptotic stimulation." Our data suggest that necrotic cell death commencing with mitochondrial damage may occur simultaneously as apoptotic changes including cell shrinkage and chromatin condensation occur downstream of the release of caspase-activating molecules from mitochondria.

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