Apaf-1-deficient *fog* mouse cell apoptosis involves hypopolarization of the mitochondrial inner membrane, ATP depletion and citrate accumulation

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To explore how the intrinsic apoptosis pathway is controlled in the spontaneous *fog* (forebrain overgrowth) mutant mice with an *Apaf1* splicing deficiency, we examined spleen and bone marrow cells from *Apaf1*^{+/+} (+/+) and *Apaf1*^{*fog/fog*} (*fog/fog*) mice for initiator caspase-9 activation by cellular stresses. When the mitochondrial inner membrane potential ($\Delta\psi$ m) was disrupted by staurosporine, +/+ cells but not *fog/fog* cells activated caspase-9 to cause apoptosis, indicating the lack of apoptosome (apoptosis protease activating factor 1 (Apaf-1)/cytochrome *c*/(d)ATP/procaspase-9) function in *fog/fog* cells. However, when a marginal (~20%) decrease in $\Delta\psi$ m was caused by hydrogen peroxide (0.1 mM), peroxynitrite donor 3-morpholinosydnonimine (0.1 mM) and UV-C irradiation (20 J/m²), both +/+ and *fog/fog* cells triggered procaspase-9 auto-processing and its downstream cascade activation. Supporting our previous results, procaspase-9 pre-existing in the mitochondria induced its auto-processing before the cytosolic caspase activation regardless of the genotypes. Cellular ATP concentration significantly decreased under the hypoactive $\Delta\psi$ m condition. Furthermore, we detected accumulation of citrate, a kosmotrope known to facilitate procaspase-9 dimerization, probably due to a feedback control of the Krebs cycle by the electron transfer system. Thus, mitochondrial *in situ* caspase-9 activation may be caused by the major metabolic reactions in response to physiological stresses, which may represent a mode of Apaf-1-independent apoptosis hypothesized from recent genetic studies.

Keywords: apoptosis, Apaf-1, caspase-9, mitochondria, citrate, ATP

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Introduction

Activation of initiator caspase-9 determines the onset of the mitochondrion-linked intrinsic pathway of apoptosis in response to diverse cellular stresses. In the original model, formation of ‘apoptosome’ comprising apoptosis protease activating factor 1 (Apaf-1), cytochrome *c* and procas-

pase-9 is assumed to be the only mechanism to convert procaspase-9 to the active form caspase-9 in the cytosol [1–4]. The association between Apaf-1 and procaspase-9 is mediated by the caspase-recruitment domain (CARD) of each protein, where cytochrome *c* and ATP/dATP are required as cofactors [2, 5–7]. According to this model, translocation of cytochrome *c*, the ion carrier associated with the complexes III and IV of the mitochondrial electron transfer system, to the cytosol is an essential event for the apoptosis cascade activation.

The mechanism of cytochrome *c* translocation has been explained by a phenomenon termed mitochondrial membrane permeabilization (MMP) [8]. Proapoptotic Bcl-2 family proteins, the effector molecules of various death signal pathways, induce a full permeability transition or a short-lived permeability pore opening of the outer

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Abbreviations: MMP (mitochondrial membrane permeabilization); $\Delta\psi$ m (mitochondrial inner membrane potential); Apaf-1 (apoptosis protease activating factor 1); MEF (mouse embryonic fibroblasts); STS (staurosporine); Sin-1 (3-morpholinosydnonimine)

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membrane, depending on the assay systems [8-10]. The former is associated with failure of the inner membrane potential ($\Delta\psi_m$) and matrix swelling, whereas the latter is not. Substantial remodeling of the mitochondrial ultra-structure was also reported [11]. Reactive oxygen species (ROS) excessively produced by the respiratory chain can also cause the progressive mitochondrial damage leading to apoptosis [12]. All of the proposed cytochrome *c*-releasing mechanisms, however, remain hypothetical, and fail to provide a physiological basis underlying naturally occurring apoptosis.

Apaf1-null mice frequently exhibit morphological defects in the forebrain [13, 14]. However, a fraction of *Apaf1*^{-/-} animals survive, suggesting that Apaf-1 plays its role in limited physiological contexts [14]. Either Apaf-1-deficient or caspase-9-deficient thymocytes could induce varied apoptotic responses to irradiation and dexamethasone [15]. Apaf-1-deficient primary myoblasts could activate caspase-9, which suggested that coupling of caspase-9 with Apaf-1 is cell type-specific [16]. A similar observation was made with *Drosophila melanogaster* lacking ARK, the fly homologue of Apaf-1 [17]. Apoptosome-independent, but Apaf-1-dependent, caspase activation was detected in thymocytes having a mutant cytochrome *c* [18]. Apoptosis-inducing mechanisms distinct from the apoptosome model deserve more extensive investigation by various approaches.

In fact, procaspase-9 is able to homo-dimerize to gain its enzyme activity in the absence of Apaf-1 as evidenced by bacterial expression systems, *in vitro* translation and biochemical analyses [7, 19-22]. More importantly, procaspase-9 is dimerized by higher concentrations of kosmotropes, salts able to stabilize proteins, such as 1 M citrate [21, 22]. Citric acid is the first product in the Krebs (citric acid) cycle whose reactions are controlled by the electron transfer system maintaining $\Delta\psi_m$ for ATP synthesis. Furthermore, a population of procaspase-9/caspase-9 and other caspases pre-exist in the intermembrane space of mitochondria and participate in apoptosis [23-25]. As we showed, a level of oxidative stress that causes a hypoactive $\Delta\psi_m$ condition leads to intra-mitochondrial caspase-9 activation, showing a sign of dimerization [26] and cytosolic translocation which can be prevented by Bcl-2 [25, 26].

Apaf1^{fog/fog} (*fog/fog*) mice bear an *Apaf1* deficiency due to mis-splicing between exons 3 and 4 of the transcript, and exhibit brain phenotypes similar to *Apaf1*^{-/-} mice, but live to adulthood [27]. The nucleotide sequences in intron 3 (Ensemble Gene ID: ENSMUSG00000019979) indicate premature termination of the mRNA translation to produce a CARD domain-only protein corresponding to a dominant-negative type suppressor against Apaf-1 [28]. To gain deeper insight into physiologically induced apoptosis, we *ex vivo* examined *fog* mouse cells for caspase-9 induction,

focusing on changes in $\Delta\psi_m$. In addition to the Apaf-1-dependent ultimate pathway to apoptosis, cells may have a branched, flexible pathway that can deal with stresses inside the mitochondria.

Results

Detection of caspase-9 activation in fog/fog and +/+ mice

Heterozygous *Apaf1*^{fog/+} pairs were mated to obtain *Apaf1*^{+/+} (+/+) and *Apaf1*^{fog/fog} (*fog/fog*) progeny. The genotypes were assessed by RT-PCR with the intron 3-spanning primer pairs, in which the *Apaf1*⁺ and *Apaf1*^{fog} alleles appeared at the expected Mendelian ratio [27]. The *fog/fog* newborns frequently displayed exencephaly and cranioschisis corresponding to the *Apaf1* knockout (KO) mice (Figure 1A and 1B).

Spleen cells from 9 newborn mice of the L1 litter, 14 days old, were *ex vivo* examined for initiator caspase-9 activation by incubation with 0.1 mM H₂O₂ as described [26]. Conversion of the 49 kilodalton (kDa) procaspase-9 (of mouse) to the auto-processed form, 37 kDa caspase-9, was observed in all cultures 5 h after exposure to H₂O₂, but not in the control culture without H₂O₂ (Figure 1C). The 24 kDa segment of anti-poly(ADP-ribose)polymerase (PARP) also appeared in H₂O₂-induced samples, indicating the downstream caspase-3 activation. A control culture without H₂O₂ failed to show the PARP cleavage (Figure 1C, sample 2, -). A faint band of 39 kDa caspase-9 was also detectable, reflecting procaspase-9 processing by caspase-3, which increased further as the apoptosis reactions progressed [26]. Since auto-processing of procaspase-9 occurs by its activation through homo-dimerization, the 37-kDa form was thought to signify induction of the caspase-9 pathway [7, 19, 20, 22]. This initial experiment suggested that +/+ and *fog/fog* cells were equally capable of caspase-9 activation in response to the oxidative stress.

Apoptosis induction in +/+ and fog/fog spleen cells by various stimulants

We examined splenocytes from 6-week-old mice for apoptosis induction by incubation with oxidative stress inducer H₂O₂ (0.1 mM), 3-morpholinosydnonimine (Sin-1) (0.1 mM), known to cause nitrosative stress molecule peroxynitrite (ONOO⁻), and staurosporine (STS) (10 μ M), a generally used apoptosis inducer. UV-C irradiation (20 J/m²) was also carried out. Processing of procaspase-9 to caspase-9, its enzyme activity and nuclear morphological changes were monitored.

In the +/+ cultures stimulated with STS, approximately 30% of the cells showed nuclear condensation and fragmentation at 5 h (Figure 2A and 2B). Western blotting

Figure 1 Detection of procaspase-9 processing in *fog/fog* mutant mice. **(A)** Heterozygous (+/*fog*) mice were mated to screen their offspring for +/+ and *fog/fog* homozygous animals. Anterior portions of the L1-3 (*fog/fog*) and L1-7 (+/+) littermates were photographed at postnatal day 7. The morphological defects in the brain represent the *fog* mutation [27]. **(B)** Structures of the *fog* mRNA and its translation product. The exon-intron structure of the 5' terminus of the *Apaf1* gene is aligned with *Apaf1* and *fog* mRNAs. Dotted line indicates splicing. The *fog* mRNA retains the third intron by mis-splicing [27] to encode the entire CARD domain followed by 25 unrelated amino acid sequences. The *Apaf-1* domain structure is also aligned. **(C)** Procaspase-9 autoprocessing in spleen cells from the L1 littermates. Cells were incubated either with (+) or without (-) 0.1 mM of H₂O₂ for 5 h, and the proteins analyzed by Western blotting. Molecular masses of the proteins are indicated in kilodaltons (kDa). Positions of 49 kDa procaspase-9 (Procasp-9) as well as 37 and 39 kDa caspase-9 (Casp-9) are indicated. Cleaved PARP (24 kDa) is also shown. β -Tubulin was analyzed as a control protein. The 45 kDa biotinylated standard protein in lane M is marked with an arrowhead.

detected caspase-9 at 37 and 39 kDa (Figure 2C). Cleavage of PARP was also evident. A substantial increase in the Ac-LEHD-pNA cleaving (LEHDase) activity was observed, implying activation of caspase-9 and caspase-3 (Figure 2D) [29]. LEHD-CHO, a peptide inhibitor against the

caspase-9 pathway, protected +/+ cells from STS-induced apoptosis (Figure 2B and 2D). In contrast, *fog/fog* cells neither activated the death protease cascade nor exhibited an apoptotic morphology at 5 h. The Apaf-1-dependent caspase-9 activation mechanism was unquestionably vital in apoptosis induced by STS.

After exposure to H₂O₂, Sin-1 and UV-C, however, both +/+ and *fog/fog* cells were equally capable of initiating apoptosis. Nuclear fragmentation, auto-processing of procaspase-9, LEHDase activation and PARP cleavage were obvious at 5 h (Figure 2A-2C). LEHD-CHO inhibited the +/+ and *fog/fog* cell death triggered by these stimuli. Furthermore, thioredoxin (TRX), a thiol-disulfide oxidoreductase, also blocked cell death induced by these stimuli (Figure 2B and 2D), suggesting involvement of a redox regulatory system. These results implied that cells have an Apaf-1-independent caspase-9 activation mechanism inducible by varied stresses.

Oxidative stress-induced apoptosis in *Apaf1*-KO mouse fibroblasts

We next tested mouse embryonic fibroblasts (MEF) from *Apaf1*-wildtype (WT) and -KO animals for cell death induction by H₂O₂. After 5 h incubation with 0.2 mM H₂O₂, cells in both cultures manifested morphological features of apoptosis at the same efficiency (approximately 25%) (Figure 3A and 3B). The dying cell population had increased to 70% at 10 h. Auto-processed caspase-9 (37 kDa) was detected as a major product by Western blotting at 5 and 10 h. A decrease in PARP (116 kDa) and generation of the long PARP fragment (83 kDa) was also evident (Figure 3C). Not only LEHD-CHO but also membrane-permeable TRX inhibited the cell death efficiently (Figure 3B). Thus, WT and KO MEF seemed to activate a common mechanism for caspase activation in response to the less stringent oxidative stress. Presence of an Apaf-1-independent caspase-9 activation mechanism was strongly suggested.

We were able to detect a trace of apoptotic morphology in MEF after incubation with 0.1 mM H₂O₂ for 24 h. If exposed to >0.4 mM of H₂O₂, MEF detached from the basement within 1 h, long before the induction of cell death.

Apaf-1 dependency correlated with the range of $\Delta\psi_m$ damage

Using expanded cultures of bone marrow mast cells (BMMC), we assessed plasma membrane rearrangement and loss of $\Delta\psi_m$ by flow cytometry using Annexin V and 3,3'-dihexyloxacarbocyanine (DiOC₆(3)) as the probes, respectively [26, 30]. As early as 1 h after exposure to STS, a substantial (69±5%) decrease in $\Delta\psi_m$ was observed in both +/+ and *fog/fog* cells, without a significant increase in the Annexin V-positive cell population (Figure 4A and

Figure 2 Induction of the caspase-9 cascade in *+/+* and *fog/fog* cells. Spleen cells from *+/+* and *fog/fog* mice were exposed to STS and physiological stimulants. **(A)** Detection of nuclear condensation and fragmentation by Hoechst staining 5 h after exposure to H₂O₂ (0.1 mM), SIN-1 (Sin-1) (0.1 mM), STS (10 μ M) and UV (20 J/m²). The scale bar indicates 10 μ m. **(B)** Apoptotic cell population (%) determined by fluorescence microscopic pictures as shown in **(A)**. Apoptotic and non-apoptotic cells were counted for 3 views (200 cells in each view). Cultures pretreated with TRX or LEHD-CHO (denoted by LEHD) for 1 h prior to exposure to the stimulants were also analyzed. **(C)** Caspase-9 activation in *fog/fog* cells by various stresses. Normal (*+/+*) and *fog/fog* spleen cells stimulated with H₂O₂, SIN-1, STS and UV-C were analyzed for processing of procaspase-9 and PARP by Western blotting. Control untreated cell lysates are in the lanes marked (–). **(D)** Induction of LEHDase activity. A colorimetric assay was performed 5 h after stimulation. Relative activities are presented in per cent in comparison with H₂O₂-induced cells (100%). In **(B)** and **(D)**, each point represents the mean value with $\pm$ SD (error bar) in triplicate reactions. Similar results were obtained in two separate experiments.

4B). At 5 h of the drug application, an approximately 90% population of the *+/+* cultures had turned Annexin V-posi-

tive without a further decrease in $\Delta\psi_m$. In contrast, ~85% of the *fog/fog* cells remained Annexin V-negative at 5 h,

Figure 3 Apoptosis of *Apaf1*-KO MEF in response to oxidative stress. **(A)** MEF from WT and *Apaf1*-KO mice were incubated with H₂O₂ and analyzed for apoptotic changes. Nuclear Hoechst staining at indicated times in hours (h). The scale bar indicates 50 μm. **(B)** Apoptotic cell populations (%) during the time course. Each point indicates the mean SD of triplicate reactions. Shown here is one of the two experiments in which results were comparable. **(C)** Procaspase-9 processing detected by Western blotting. Positions of 49-kDa procaspase-9 (Procasp-9) and the 37- and 39-kDa fragments of caspase-9 (Casp-9) are indicated. Full-length PARP (116 kDa) and the longer cleavage product (83 kDa) were also detected. By probing with an anti-Apaf-1 antibody, WT and KO cell samples were verified.

with the $\Delta\psi_m$ level equal to that of *+/+* cells. Thus, *fog/fog* cells were resistant to STS-induced apoptosis, despite the sudden fall of $\Delta\psi_m$.

Cells stimulated by H₂O₂, Sin-1 and UV-C displayed only a minor decrease ($17\pm2\%$, $19\pm3\%$ and $24\pm1\%$, respectively) in $\Delta\psi_m$ at 1 h, and were negative for Annexin V-staining. Interestingly, nearly the entire population ($\geq 93\%$) of the *+/+* and *fog/fog* cultures became Annexin V-positive after 5 h, suggesting that Apaf-1-independent apoptosis was induced after a duration of hypo- $\Delta\psi_m$. The level of $\Delta\psi_m$ did not decline further for at least 5 h.

At 5 h of incubation with STS, both *+/+* and *fog/fog* cells contained a large amount of cytochrome *c* in the cytosol, showing concomitant loss of the protein from the mitochondria (Figure 5A). In contrast, the localization of cytochrome *c* was not altered in H₂O₂-induced apoptosis of *+/+* and *fog/fog* cells as analyzed by western blotting. The mitochondrial fractions obtained in this experiment were abundant in Cox IV, an inner membrane protein, while the cytosol fractions were free of the protein. Conversely, heat shock protein 90 (HSP90), a molecular chaperone protein, was localized in the cytosol, and only a minimum amount was detectable in the mitochondrial fractions. Together with the results in Figure 2, these data (Figures 4 and 5A)

suggested that STS caused a large loss of $\Delta\psi_m$, thereby mobilizing a large amount of cytochrome *c* to trigger the canonical Apaf-1-dependent caspase-9 activation cascade. The other inducers caused a hypo- $\Delta\psi_m$ condition, where cytochrome *c* was retained in the mitochondria, leading to caspase-9 activation through an Apaf-1-independent mechanism.

ATP depletion and citrate accumulation caused by hypo- $\Delta\psi_m$

To examine biochemical outcomes of the two different types of $\Delta\psi_m$ impairment, we performed semi-quantification of the whole cell ATP amount using a luciferase-based

Figure 4 Apaf-1-dependent apoptosis induction is characterized by hypo- $\Delta\psi_m$ caused by varied stimuli. **(A)** Flow cytometric analysis of BMMC (*+/+*, *fog/fog*) for $\Delta\psi_m$ and apoptosis. At the indicated time points (h) after stimulation with H₂O₂, SIN-1, STS and UV-C, cells were stained with DiOC₆(3) and Annexin V for detection of $\Delta\psi_m$ and plasma membrane rearrangement, respectively. Complete depolarization of $\Delta\psi_m$ was demonstrated by 5-min incubation with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at room temperature. Similar results were obtained by two independent experiments. One complete set is presented. **(B)** Numerical presentation of the two-dimensional dot plots shown in **(A)**.

Figure 5 Comparison of biochemical alterations in BMMC by H₂O₂ and STS. **(A)** Analysis of cytosol fractions for cytochrome c (Cyt c) content. Mitochondrial and cytosolic fractions were obtained from BMMC incubated for 5 h with H₂O₂ and STS as well as cells without stimulation (-). Both +/+ and *fogl/fogl* genotypes were tested. Western blots for cytochrome c, mitochondrial marker protein COX IV and cytosolic protein HSP90 are shown. **(B)** Semi-quantification of cellular ATP. ATP amount at each time point (0.5, 1, 2, 5, 7-h) is shown in relation to the amount in the control cell lysate (100%). Triangles and squares indicate cells exposed to H₂O₂ and STS, respectively. **(C)** Determination of citric acid amounts in cell extracts prepared at 3.5 h of incubation with H₂O₂ and STS. Citrate concentration is presented in mM. Experiments in B and C were performed three times with different pairs of +/+ and *fogl/fogl* mice. The mean values and standard deviations (error bars) are indicated.

assay (Figure 5B). In STS-stimulated cells of +/+ and *fogl/fogl*, ATP amount was not significantly altered by the sudden fall of $\Delta\psi_m$ (at 1 h). Instead, we observed a gradual decline of the ATP level to approximately 60% at 5 h, consistent with a recent study [31]. Given that $\Delta\psi_m$ disruption means shutdown of the mitochondrial ATP-producing function of the respiratory chain on the inner membrane, this result indicated that most of the cellular ATP-consuming reactions also stopped. On the contrary, H₂O₂ (0.1 mM) caused a significant decrease in ATP, reaching 5% of the control level 2 h after incubation, or 3 h before the onset of apoptosis. This ATP depletion may have resulted from the imbalance between vital ATP consumption in the pre-apoptotic phase and impaired ATP production due to the $\Delta\psi_m$ hypo-polarization.

We next carried out citric acid quantification by an enzyme-based reaction (Figure 5C). The measured citrate in the control +/+ cell extract was 0.41 ± 0.06 mM, within the range of the normal physiological concentration, 0.1–1 mM [32]. At 3.5 h of incubation with STS, a significant decrease in citric acid (to 20% of the normal level, 0.078

mM) was observed, suggesting cleavage of citrate by citrate lyase, a cytosolic enzyme, after mitochondrial swelling. Conversely, H₂O₂ caused a 3.7-fold increase in citrate to 1.51 ± 0.39 mM, implying citrate accumulation in the mitochondria due to the feedback suppression of the Krebs cycle by the hypo-polarized electron transfer system [33]. *fogl/fogl* cells displayed comparable decrease or increase in citrate 3.5 h after incubation with STS or H₂O₂.

Activation of caspase-9 associated with mitochondria

We performed cell fractionations to investigate the site of initial activation of caspase-9 in stress-induced apoptosis. Mitochondrial and cytosolic fractions were obtained in the course of H₂O₂-induced BMMC apoptosis. The purity of these fractions was confirmed by the contents of COX IV and HSP90 as described above. Western blotting showed generation of auto-processed caspase-9 (37 kDa) in the mitochondria 2 h after the input of H₂O₂ (Figure 6B). In the cytosol fractions, an increase in the mature protein band became obvious at 5 h, leading to a further enhancement at 7 h. LEHDase activity was first detectable at 2 h in the mitochondrial fraction from either +/+ or *fogl/fogl* cells (Figure 6A). The enzyme activity in the mitochondria slightly

Figure 6 Activation of caspase-9 associated with mitochondria. **(A)** Levels of LEHDase activity in the mitochondrial (Mt) and cytosol (Cyt) fractions. Cells were fractionated at indicated time intervals after stimulation with H₂O₂. Enzyme activity at each time point (h) is indicated in relation to the maximum activity in the cytosol of the H₂O₂-induced +/+ cells at 7 h (100%). Mean values SD (error bars) were calculated from two separate experiments. **(B)** Western blot analysis for procaspase-9 processing in the mitochondrial and cytosolic fractions. Five portions of each mitochondrial fraction were loaded in parallel with one portion of the corresponding cytosol fraction. The contents of COX IV and HSP90 were also analyzed to verify the purity of these fractions.

declined at 5 h. In contrast, cytosolic LEHDase activation became evident at 5 h, with a further increase (2.5-fold) at 7 h. These data indicated that under such conditions the initial caspase-9 activation was associated with mitochondria, and that the cytosolic caspase induction and amplification occurred later on, in both *+/+* and *fog/fog* cells.

Discussion

Using the splenocytes and BMMC from normal and *fog/fog* mice, we detected two distinguished modes of mitochondrially controlled apoptosis. STS evidently induced the Apaf-1-dependent pathway associated with $\Delta\psi_m$ disruption and cytochrome *c* release, supporting the apoptosome model. On the other hand, both *+/+* and *fog/fog* cells were able to induce procaspase-9 auto-processing and the downstream reactions in response to varied stimuli related to physiological regulators: H_2O_2 representing cellular ROS; Sin-1 that releases peroxynitrite ($ONOO^-$) involved in cardiovascular and other pathologies via its oxidative and nitrosative actions [34, 35]; and UV-C irradiation causing DNA damage and p53 activation [36]. With *Apaf1*-null MEF, we also detected induction of the Apaf-1-independent pathway by oxidative stress.

Unlike the general thought of STS, a recent comprehensive study in chemical biology unequivocally characterized it as a nonspecific kinase inhibitor [37]. The marine mollusk alkaloid lamellarin D acts as a strong inducer of apoptosis by causing $\Delta\psi_m$ disruption and permeability transition pore opening [38]. STS might also directly destroy $\Delta\psi_m$ to cause cytochrome *c* release and mitochondrial swelling. The apoptosome model may be more relevant to apoptosis induced by acute stressors including ischemia reperfusion [39] and arsenic trioxide [40] than that by physiological stresses caused by homeostatic imbalance. Of note, these acute stimuli lead to a high level production of ROS to destroy $\Delta\psi_m$ [39, 40].

Apaf-1-independent caspase-9 activation occurred under the hypo- $\Delta\psi_m$ condition directly indicative of impairment of the ATP-synthesizing function. Indeed, we detected a large decrease in ATP amount upon the hypo-polarization. Despite the inner membrane damage, cellular ATP-consuming reactions may have continued in the H_2O_2 -stimulated cells. Since the apoptosome formation requires ATP, this ATP depletion might be suppressive of the Apaf-1-dependent pathway. On the other hand, (d)NTPs at the physiological levels block cytochrome *c* from Apaf-1-binding *in vitro* [31]. In agreement with the recent result, our experiment also detected a modest decrease in ATP after the $\Delta\psi_m$ disruption by STS. Apoptosis induction may be influenced in multiple mechanisms by ATP.

The Apaf-1-independent cell death induction by the

varied stimuli was attenuated by TRX, implying involvement of ROS generated from the respiratory chain. Intriguingly, we detected accumulation of citric acid in BMMC manifesting hypo- $\Delta\psi_m$, suggesting the feedback regulation of the Krebs cycle by the respiratory chain [33]. More specifically, $[O_2]^-$ produced from the respiratory chain inactivates aconitase, an iron-sulfur (Fe-S) protein that catalyzes hydration of citrate at the beginning of the Krebs cycle, and thereby causes citrate accumulation in the matrix [12]. Given the nucleus/cytoplasm volume ratio, 7/10 [41], and the mitochondria/cytosol ratio, 6.2/100, the maximum citrate concentration in the total mitochondrial volume would reach ~125 mM. The matrix contains yet a higher concentration of citrate which is emitted to the intermembrane space, where procaspase-9 molecules exist. Mitochondrial local microenvironments with this citrate accumulation may increase the chance of procaspase-9 dimer formation (Figure 7), as *in vitro* studies showed efficient procaspase-9 homo-dimerization and activation by 1 M ammonium citrate or 0.7-1 M sodium citrate without an alteration of its substrate specificity [21, 22].

Figure 7 Schematic presentation of the mitochondrial biochemical alterations in the course of Apaf-1-independent caspase-9 activation. The mitochondrial inner membrane is illustrated with the electron transfer system (complexes I-IV), ATP synthase and citrate transporter (oval) [33]. A part of the Krebs cycle in the matrix is depicted. The outer membrane with pores permeable to the metabolites is also shown. Biochemical changes revealed by this study are highlighted in magenta. Bold arrows indicate an increase (upward) or decrease (downward) in each physiological parameter. Bar-headed lines indicate inhibition. The hypo- $\Delta\psi_m$ condition and the results with thioredoxin (TRX) predict free radical ($[O_2]^-$) production by which aconitase is inactivated [12]. Dimerization of procaspase-9 by a topical increase in citrate is hypothesized. Cytosolic translocation of caspase-9 is inhibited by Bcl-2 [24, 26], although the mechanism has not been identified (blue box).

The newly proposed pathway may respond to gradually increasing physiological stresses and apoptosis signals before the matrix eventually swells by disruption of $\Delta\psi_m$. Due to the technical difficulty in detection of hypo- $\Delta\psi_m$ and intra-mitochondrial caspase-9 activation, this pathway might have been overlooked or considered to be a non-apoptotic mechanism. Furthermore, although several reports unambiguously showed release of active caspases from mitochondria to the cytosol [23, 24, 26], the mechanism is yet to be determined, as is the cytochrome *c*-releasing mechanism [8]. In addition to the functions of pro-apoptotic Bcl-2 family proteins [8, 9], ROS-mediated permeability transition pore opening of the inner and outer membranes [12, 42] may be underscored. Supporting the proposition that coupling of caspase-9 with Apaf-1 is cellular context-dependent [16], this study suggests that the range of $\Delta\psi_m$ damage is an important parameter for Apaf-1-dependent and independent apoptosis pathway usage. In conclusion, we detected Apaf-1-dependent apoptosis by STS in *+/+* cells, and Apaf-1-independent apoptosis by varied physiological stimuli in *+/+* and *fog/fog* cells. The Apaf-1-independent apoptosis seems to involve mitochondrial major metabolic reactions including the electron transfer system and Krebs cycle.

Materials and Methods

Fog mice

Heterozygous *fog/+* pairs of the fog mice (Jackson Lab Strain Name: STOCK Apaf1*fog*/J) were mated to obtain the *fog/fog* and *+/+* progeny mice. At 2 weeks after birth, blood samples (10 μ l) were obtained from the tail vein for small-scale RNA extraction (RNAqueous, Ambion) for RT-PCR with primer pairs, *fog1/fog13* and *fog1/fog14* [27]. Total RNA preparation from cell cultures was carried out with RNeasy (Qiagen).

Cell cultures and stress induction

Cells from the spleen and bone marrow were incubated in a buffer (0.14 M NH_4Cl , 17 mM Tris-HCl, pH 7.2) to remove erythrocytes. After two washes, splenocytes were incubated with RPMI1640 supplemented with 1% sodium pyruvate, 1% glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM non-essential amino acids, 10 mM HEPES and 10% fetal bovine serum (FBS). Cells from the bone marrow were cultured in the same medium supplemented with 0.1% 2-mercaptoethanol (2ME) and 4 ng/ml of IL-3 as described [43] to obtain c-kit/Fc ϵ RI double-positive BMDC at 80% homogeneity as judged by flow cytometry. For apoptosis induction, cells were incubated with H_2O_2 (0.1 mM), Sin-1 (Dojin) (0.1 mM) or STS (Sigma) (10 μ M) in the medium without 2ME for 5 h. UV-C (20 J/m²) irradiation [36] and a subsequent 5 h of incubation were also performed. MEF were cultured as described [13], and examined for H_2O_2 (0.2 mM)-induced cell death in Dulbecco's modified minimum essential medium (with high glucose) supplemented with 20% FBS. Endotoxin-free recombinant TRX and cell-permeable caspase-9 inhibitor LEHD-CHO (caspase-9 inhibitor II) were from Calbiochem.

Cell fractionation and purification of mitochondria

Mitochondria were isolated and treated as described [26]. In brief, washed BMDC (10^7) were disrupted with 40 strokes in a loosely fitted Dounce homogenizer in 3 volumes of isolation buffer: 20 mM Hepes, pH 7.4; 10 mM KCl; 1.5 mM MgCl_2 ; 1 mM EDTA; 1 mM EGTA; 1 mM dithiothreitol (DTT); 250 mM sucrose; and protease inhibitors. After two-cycle clarification at $2\,500 \times g$ for 5 min, supernatant was centrifuged at $12\,000 \times g$ for 30 min to obtain the cytosol and mitochondrial fractions. If required, the cytosol fraction was concentrated by centrifugal ultrafiltration units (Vivaspin 2, MWCO 5000, Vivascience).

Western blotting

For sample preparation, spleen cells (1×10^7) and BMDC (1×10^6) were washed in an ice-cold buffer (0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA), lysed in 200 μ l of SDS-PAGE sample buffer and boiled immediately [26]. Protein quantification was with the BCA protein assay reagent (Pierce). Total cell lysate (100 μ g of proteins) was loaded in a slot. The mitochondrial and cytosol fractions were loaded at a ratio of 5:1, by which approximately 20 μ g of proteins were separated in one lane. Separating gel (1.5 mm thick) was prepared with 9% or 11% acrylamide. Biotinylated SDS-PAGE standards (broad range, Bio-Rad) were used for size determination. A mouse-specific (#9504) anti-caspase-9 antibody and an anti-PARP antibody (#9542) were obtained from Cell Signaling Technology. Antibodies against anti-COX IV (ab16056, Abcam), HSP90 (ab1429, Abcam) and β -tubulin (Sigma-Aldrich) were used for detection of the loading control proteins. The blotting membrane was exposed to an instant film (FP-3000B, Fuji) with an ECL Mini Camera (Amersham Biosciences).

Cell staining and flow cytometry

Nuclei were stained with Hoechst 33342 (ICN Biomedicals) to obtain apoptotic cell ratios by counting 500 cells in one experiment [26]. For flow cytometry, cells were stained with DiOC₆(3) (Aldrich) and biotinylated annexin V/streptavidin-allophycocyanin (BD Biosciences), and analyzed by FACS Calibur (BD Biosciences). Carbonyl cyanide 3-chlorophenylhydrazone (Wako), a protonophore abbreviated CCCP, was used as $\Delta\psi_m$ disruptor.

Caspase enzyme assay

Ac-LEHD-pNA (Calbiochem), a substrate designed for caspase-9, is in fact cleaved not only by caspase-9 but also by caspase-3 [29], and the enzyme activity determined by the colorimetric assay is referred to as 'LEHDase'. Mitochondrial and cytosolic fractions in the assay buffer containing Tris-HCl (50 mM, pH 7.6), DTT (10 mM), phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (1 μ g/ml), aprotinin (1 μ g/ml), and pepstatin A (1 μ g/ml) were incubated with the substrate (0.2 mM) for 10 min at 37 °C. Isolated mitochondria were permeabilized with 0.1% Tween-20 prior to the enzyme reaction. Absorbance at 405 nm was measured.

Quantification of ATP and citrate

We semi-quantified cellular ATP amounts by a luciferase enzyme assay system using the CellTiter-Glo Luminescent Assay kit (Promega). Citrate concentration was determined with K-CITR (Megazyme, Wicklow, Ireland), an assay system with citrate lyase and NADH. Aliquots of deproteinized cellular extracts (50 μ l from 10^8 cells) were subjected to the enzyme reaction, and concentrations (mM) calculated from the decrease in absorbance at 340 nm.

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