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The Apoptotic Protease-Activating Factor 1-Mediated Pathway of Apoptosis Is Dispensable for Negative Selection of Thymocytes

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Negative selection is a process to delete potentially autoreactive clones in developing thymocytes. Programmed cell death or apoptosis is thought to play an important role in this selection process. In this study, we investigated the role of apoptotic protease-activating factor 1 (Apaf1), a mammalian homologue of CED-4, in programmed cell death during the negative selection in thymus. There was no developmental abnormality in thymocytes from newborn Apaf1−/− mice in terms of CD4 and CD8 expression pattern and thymocyte number. Clonal deletion by endogenous male H-Y Ag of Apaf1-deficient thymocytes with transgenic expression of H-Y Ag-specific TCRs (H-Y Tg/Apaf1−/− thymocytes) was normally observed in lethally irradiated wild-type mice reconstituted with fetal liver-derived hemopoietic stem cells. Clonal deletion induced in vitro by a bacterial superantigen was also normal in fetal thymic organ culture. Thus, Apaf1-mediated pathway of apoptosis is dispensable for the negative selection of thymocytes. However, H-Y Tg/Apaf1−/− thymocytes showed partial resistance to H-Y peptide-induced deletion in vitro as compared with H-Y Tg/Apaf1+/− thymocytes, implicating the Apaf1-mediated apoptotic pathway in the negative selection in a certain situation. In addition, the peptide-induced deletion was still observed in H-Y Tg/Apaf1−/− thymocytes in the presence of a broad spectrum caspase inhibitor, z-VAD-fmk, suggesting the presence of caspase-independent cell death pathway playing roles during the negative selection. We assume that mechanisms for the negative selection are composed of several cell death pathways to avoid failure of elimination of autoreactive clones. The Journal of Immunology, 2002, 168: 2288–2295.

Apoptosis or programmed cell death (PCD) is essential for the normal development of the body and the precise regulation of homeostasis in multicellular organisms (1). Apoptosis is also critical for the development and homeostasis of T cells (2, 3). In the thymus, CD4Cd8 double-positive (DP) thymocytes bearing TCRs that fail to recognize the self MHC molecules die rapidly through a process termed death by neglect. In contrast, recognition of self MHC structure with bound peptide can trigger either functional differentiation (positive selection) or apoptosis (negative selection) of DP cells. If positively selected, immature DP thymocytes develop into mature single-positive (SP) T cells expressing either CD4 or CD8. DP thymocytes bearing TCRs that strongly react with relatively abundant thymic self Ags undergo negative selection, i.e., the clonal deletion of potentially autoreactive T cells. Thus, the process of negative selection results in the PCD of over 97% of developing thymocytes (4, 5). The molecular mechanisms of apoptosis involved in these thymic selection processes, however, remain unclear. In the periphery, self-reactive T cells as well as Ag-stimulated mature T cells are deleted by a mechanism of activation-induced cell death, which is mainly mediated by Fas-mediated apoptosis (6). Apoptosis in these situations prevents autoimmune disease and inappropriate accumulation of activated lymphocytes.

A recent advance has shown that mitochondria play essential roles in apoptosis (7–10). While mitochondria produce metabolic energy in the form of ATP, they contain and release proteins that are involved in the apoptotic cascade, such as cytochrome c (cyto c) and some of caspases (11). Cyto c, an essential component of the respiratory chain of the mitochondria, is released in response to various apoptotic stimuli (12, 13) and binds the apoptotic protease-activating factor 1 (Apaf1), a mammalian homologue of CED-4, leading to the formation of apoptosome, which then proteolytically activates caspase 9. The activated caspase 9 cleaves the downstream caspases, including caspases 3, 6, and 7, to execute apoptotic cell death by digesting essential cellular proteins (14, 15). Thus, deficiency of one of the essential components of the mitochondrial apoptotic pathways renders the cells remarkably resistant to apoptotic stimulation, as shown in gene-disrupted mice (16–21). Apaf1-deficient (Apaf1−/−) mice die perinatally, and those embryos have defects in PCD in various tissues whose development is regulated by PCD, including removal of the interdigital webs, formation of the palate, control of the number of neurons, and development of the lens and retina (19). Apaf1−/−...
obtained from H-Y Tg/H11001 Apaf1 protocol is shown in Fig. 4 mice were used for analyses at least 6 wk after transfer. A schematic pro-
terns was normally executed in Apaf1-deficient thymocytes, demon-
ning that Apaf1-dependent apoptotic pathway is dispensable for PCD during the negative selection process. However, we also showed that Apaf1-deficient thymocytes are more resistant to the peptide-induced cell death in vitro, implicating Apaf1-mediated apoptotic pathway in the negative selection of thymocytes. In addi-
tion, we demonstrate that Apaf1-independent caspase activation and cell death that were not inhibited by a broad spectrum caspase inhibitor, z-VAD-fmk, occurred during the peptide-induced cell death in vitro. Taken together, these data indicate that the cell death mechanisms of negative selection are composed of several pathways, which presumably play synergistic and mutually compensatory roles.

Materials and Methods

Mice

The mice bearing Tg TCR specific for male H-Y Ag peptide on H-2D\(^b\) (H-Y Tg mice) were provided from the Agen Institute (Toronto, Ontario, Canada) and maintained on C57BL/6 background. Mice positive for the Tg were typed by clonotypic TCR expression using the specific mAb (T3.70; specific for \(\alpha\)-chain of H-Y TCR) (24). Apaf1\(^{-/-}\) mice were generated as described previously (19), and were backcrossed into the C57BL/6 background more than six times before crossing with H-Y Tg mice. H-Y Tg Apaf1\(^{+/+}\) mice were generated by crossing H-Y Tg mice with Apaf1\(^{+/+}\) male; H-Y Tg mice were also used for crossing to obtain mice with the Y chromosome derived from C57BL/6 (but not from 129) background. Mice were confirmed for H-2\(^b\) phenotype. C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). These mice were maintained in a pathogen-free condition.

Genotyping

Genotyping of Apaf1\(^{-/-}\), Apaf1\(^{+/+}\), and Apaf1\(^{+/+}\) mice or fetuses was performed using PCR analysis of tail DNA. Two PCR primer sets were used for genotyping. One primer set for detecting the wild-type allele is 5'-CCA TTC CTG GTC TCT TGT AAG A-3' and 5'-AAC ACG GAG GGC GTC TTT T-3'. The other primer set for detecting the mutant type allele is 5'-GGG CCA CTC GTT ATG GGC GCT ATC TCC A-3' and 5'-CAC TCT ATG GTC CAG GCT ATC T-3'.

Generation of lethally irradiated wild-type mice reconstituted with fetal liver-derived hematopoietic stem cells

H-Y Tg/Apaf1\(^{+/+}\) or Apaf1\(^{+/+}\) fetuses at embryonic day (E) 14.5 were obtained from H-Y Tg/Apaf1\(^{+/+}\) or Apaf1\(^{+/+}\) mice intercrosses. The sex of fetuses was determined individually under a microscope, and liver cell suspensions were prepared from each fetus. Eight-week-old male or female C57BL/6 mice were irradiated (900 rad), and approximately 2–5 \(\times\) 10\(^6\) of fetal liver cells from each fetus were transferred i.v. to each irradiated, sex-matched mouse. Thymocytes isolated from these fetal liver-transferred chimeric mice were used for analyses at least 6 wk after transfer. A schematic protocol is shown in Fig. 4A.

Fetal thymic organ culture

FTOC were performed as described previously (23). Briefly, the thymic lobes were obtained from Apaf1\(^{-/-}\) or Apaf1\(^{+/+}\) fetuses at E14.5, and cultured on polycarbonate filters (pore size, 4.5 \(\mu\)m; Millipore, Bedford, MA) floating on complete RPMI 1640 medium supplemented with 10% FCS in a humid atmosphere with 5% CO\(_2\). The lobes were cultured for 5 days, and followed by further cultivation in the presence or absence of 1 \(\mu\)g/ml staphylococcal enterotoxin B (SEB; Sigma, St. Louis, MO) for 2 days. For harvesting, lobes were ground between frosted glass slides in PBS, washed, and used for flow cytometric analysis.

Flow cytometric analysis and Abs

mAbs used for flow cytometric analysis were FITC-conjugated T3.70 mAb, PE- or allophycocyanin-conjugated anti-CD8 mAb (2,43; BD Pharmingen, San Diego, CA), allophycocyanin-conjugated anti-CD4 mAb (L374; BD Pharmingen), allophycocyanin-conjugated anti-CD3e mAb (I45-2C11; BD Pharmingen), FITC-conjugated anti-V\(\beta\)6 mAb (44-22-1), and FITC-conjugated anti-V\(\beta\)8 mAb (F23.1; BD Pharmingen). Freshly isolated or cultured cells were stained with various combinations of mAbs before analysis with a FACS Calibur flow cytometer and CellQuest program (Becton Dickinson, Franklin Lakes, NJ). In some experiments, cell suspensions were stained with propidium iodide (PI) just before analysis to detect and exclude dead cells. Mitochondrial transmembrane potential (\(\Delta\psi\)m) was measured by staining the cells with potential sensitive dye, 3,3’-diethyloxadecarbocyanine iodide (DiOC\(_3\)(3) (Molecular Probes, Eugene, OR), as described elsewhere (19).

Peptide-induced deletion assay in vitro

Single cell suspension (\(5 \times 10^5\)) of thymocytes from female fetal liver-transferred chimeric mice was cultured with 10 \(\mu\)M H-Y Ag peptide (sequence Lys-Cys-Ser-Arg-Asn-Arg-Gln-Tyr-Leu (25)) (Generned Synthesis, South San Francisco, CA) in the presence or absence of 100 \(\mu\)M z-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk; Kamiya Biomedical, Seattle, WA) for 24 h in a 96-well plate. After culture, thymocytes were harvested and stained with FITC-conjugated T3.70 mAb, PE-conjugated anti-CD8 mAb, and PI for evaluation of dead cells, or with FITC-conjugated T3.70 mAb, allophycocyanin-conjugated anti-CD8 mAb, and PhiPhiLux G2D2 (26) (MBL, Nagoya, Aichi, Japan) for evaluation of cells with active caspase 3, and followed by flow cytometric analysis.

T cell proliferation assay

Nylon wool-nonadherent lymph node cells containing 3 \(\times 10^9\) T cells were used as a stimulator. The spleen cells from male or female C57BL/6 mice were cultured for 4 days, followed by harvesting 20 h later.

Measurement of cyto c release

Thymocytes from wild-type mice with or without peptide stimulation were homogenized in ice-cold preparation buffer (10 mM Tris-HCl, pH 7.5, and 0.3 M sucrose with protease inhibitors) and supernatants collected after centrifugation at 10,000 \(\times\) g for 60 min. The amounts of cyto c in the supernatants were measured by cyto c ELISA assay kit (MBL), according to the manufacturer’s direction.

Western blot analysis of caspase activation

Thymocytes from fetal liver-transferred chimera mice (Apaf1\(^{-/-}\) or Apaf1\(^{+/+}\)) with or without peptide stimulation were lysed with 3-[3-chol-

amidopropyl]dimethylammonio]-1-propanesulfonate buffer (Cell Signal-
ning Technology, Beverly, MA), electrophoresed, and transferred onto a nylon membrane. Caspases 3, 6, and 7 were visualized with anti-caspase Abs (Cell Signaling Technology).

Results

Normal development of Apaf1-deficient thymocytes

Apaf1\(^{-/-}\) mice exhibited abnormalities in brain during embryogenesis and thus died perinatally (19, 20), although a few mice with a milder mutant phenotype (ectopic masses on the forehead) survived until about day 10 (19). Apaf1\(^{-/-}\) thymocytes from these mice are strikingly resistant to a wide range of apoptotic stimuli, such as dexamethasone, gamma irradiation, and anticancer drugs, except for Fas ligation (19). However, flow cytometric analysis of thymocytes of newborn (day 10) Apaf1\(^{-/-}\) mice showed that the thymic development of these mice appeared to be largely normal in terms of expression patterns of CD4 and CD8 (Fig. 1). There
was no increase in thymocyte number as compared with Apaf1+/+ and Apaf1+/− littermates. In addition, there was no significant difference in the total number of thymocytes or the expression patterns of CD4 and CD8 between Apaf1+/−/Rag−/− and Apaf1−/−/Rag−/− somatic chimeras (19); 8.9 ± 0.9 × 107 and 83.5 ± 5.9% in Apaf1+/−/Rag−/− and 8.3 ± 1.5 × 107 and 82.1 ± 7.1% in Apaf1−/−/Rag−/− somatic chimeras, total thymocyte number, and the percentage of CD4+CD8+ thymocytes, respectively. Non-Tg Apaf1+/− or Apaf1−/− fetal liver-transferred chimeric mice also showed similar results (data not shown). Thus, the Apaf1-mediated apoptotic pathway is dispensable for normal thymic development.

Antigenic peptide-induced dissipation of ΔΨm and cyt c release

We next investigated whether stimulation of thymocytes with physiological antigenic peptide leads to mitochondrial alterations, the upstream events of the Apaf1-mediated apoptotic pathway. To do so, we took advantage of the mice bearing Tg TCR specific for the upstream events of the Apaf1-mediated apoptotic pathway. To investigate whether Apaf1-mediated apoptotic pathway is involved in PCD during the negative selection, we first examined the clonal elimination of thymocytes from Apaf1−/− fetuses induced by addition of a bacterial superantigen, SEB, into FTOC, which has been used as a model of negative selection based on apoptotic clonal deletion (23). As shown in Fig. 3, SEB-reactive Vβ8+ cells in CD4+CD8− population were eliminated similarly in both Apaf1+/− and Apaf1−/− thymocytes by addition of SEB (11–14.9% to 4.3–7.2% in Apaf1+/− and 10.8–13.8% to 4.4–6.6% in Apaf1−/− in three independent experiments). Percentage of SEB nonreactive Vβ6− cells was not affected. Thus, Apaf1 deficiency did not affect the superantigen-induced elimination of thymocytes in this system of negative selection.

Normal negative selection of Apaf1−/− thymocytes bearing H-Y TCRs in fetal liver-transferred chimeric mice

We then examined the negative selection by endogenous self Ag in Apaf1−/− thymocytes. To do so, we generated and analyzed C57BL/6 radiation chimeras reconstituted with fetal liver-derived hematopoietic stem cells from H-Y Tg mice with Apaf1+/− or Apaf1−/− genotype (Fig. 4A; H-Y Tg/Apaf1+/− or Apaf1−/− fetal liver-transferred chimeric mice, respectively). In male mice, the showed a peptide dose-dependent dissipation of the ΔΨm, as shown in Fig. 2A. However, the degree of dissipation was unexpectedly small when approximately 70% of the thymocytes were H-Y TCR+ (T3.70) and CD8+ (data not shown). We also examined the release of cyt c, the trigger of the initiation of the Apaf1-mediated apoptotic pathway in response to the peptide stimulation. The peptide stimulation induced the release of cyt c in a dose-dependent manner (Fig. 2B). Thus, it appears that thymocyte stimulation with relevant Ag induces mitochondrial alterations.

SEB-induced deletion of Apaf1-deficient thymocytes

Since mitochondrial alteration occurred in response to the peptide stimulation, an impairment of negative selection in the absence of Apaf1, an immediate downstream molecule of the mitochondrial damages, was examined. To investigate whether Apaf1-mediated apoptotic pathway is involved in PCD during the negative selection, we first examined the clonal elimination of thymocytes from Apaf1−/− fetuses induced by addition of a bacterial superantigen, SEB, into FTOC, which has been used as a model of negative selection based on apoptotic clonal deletion (23). As shown in Fig. 3, SEB-reactive Vβ8+ cells in CD4+CD8− population were eliminated similarly in both Apaf1+/− and Apaf1−/− thymocytes by addition of SEB (11–14.9% to 4.3–7.2% in Apaf1+/− and 10.8–13.8% to 4.4–6.6% in Apaf1−/− in three independent experiments). Percentage of SEB nonreactive Vβ6− cells was not affected. Thus, Apaf1 deficiency did not affect the superantigen-induced elimination of thymocytes in this system of negative selection.

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H-Y TCR is self-reactive and results in negative selection of H-Y TCR⁺ (T3.70⁺) cells during thymocyte development. Conversely, in female mice, H-Y TCR⁺ thymocytes are positively selected and develop into mature CD8⁺ SP cells. Flow cytometric analysis of thymocytes from these fetal liver-transferred chimeric mice showed that negative selection of Tg-positive T3.70⁺ CD8⁺ thymocytes in male mice was complete in the Apaf1⁻/- background (Fig. 4B, top). Positive selection in female mice was likewise unchanged in thymocytes from H-Y Tg/Apaf1⁻/- fetal liver-transferred chimeric mice (Fig. 4B, bottom). These results suggest that PCD pathway for the elimination of self-reactive clones during thymic negative selection does not require Apaf1.

Lack of self-reactive T cell population in the periphery of H-Y Tg/Apaf1⁻/- fetal liver-transferred chimeric mice

In male H-Y Tg mice, there are many T3.70⁺ CD8⁺ T cells in the periphery. We also found T3.70⁺ CD8⁺ T cells in the periphery of male H-Y Tg/Apaf1⁻/- fetal liver-transferred chimeric mice. Although these T3.70⁺ CD8⁺ T cells in male H-Y Tg mice reportedly develop extrathymically and are unresponsive to male H-Y Ag (24, 27, 28), it was of importance to examine whether self-reactive T cells were actually eliminated from periphery of the H-Y Tg/Apaf1⁻/- fetal liver-transferred chimeric mice. To address this question, we examined anti-male Ag response of T3.70⁺ CD8⁺ lymph node T cells from the fetal liver-transferred chimeric mice and compared it with those from male or female H-Y Tg mice. In H-Y Tg mice, peripheral T3.70⁺ CD8⁺ T cells from female mice showed strong proliferative response to male spleen cells, while those from male mice did not respond to male cells, confirming that T3.70⁺ CD8⁺ cells with reactivity to self male Ag are eliminated in thymus by negative selection (Fig. 5). Similarly, peripheral T cells from female H-Y Tg/Apaf1⁻/- or Apaf1⁻/- fetal liver-transferred chimeric mice showed strong proliferative response to male Ag. However, no proliferative response to male Ag was observed in cells from either Apaf1⁻/- or Apaf1⁻/- male chimeric mice. These results showed that self-reactive T cells are virtually absent in the periphery of male H-Y Tg/Apaf1⁻/- fetal liver-transferred chimeric mice, indicating complete negative selection in the thymus of these mice.

Apaf1-dependent and Apaf1-independent cell death of thymocyte induced by antigenic peptides

Normal negative selection of Apaf1-deficient thymocytes in SEB-induced and H-Y Ag-induced negative selection system was unexpected, when involvement of caspase 3 activation in the TCR-induced negative selection both in vitro and in vivo (29, 30) and the mitochondrial alterations by antigenic stimulation (Fig. 2) are taken into consideration. Therefore, to examine whether or not the susceptibility of Apaf1⁻/- thymocytes to TCR stimulation nonetheless differs from that of Apaf1⁺/- thymocytes, we performed

![Diagram](http://www.jimmunol.org/Downloadedfrom)
antigenic peptide-induced deletion assay in vitro using thymocytes from female H-Y Tg/ Apaf1−/− fetal liver-transferred chimeras, as a surrogate for negative selection in vivo. Addition of H-Y peptide, which is specifically recognized by H-Y TCRs, to the thymocyte culture induces the deletion of peptide-specific T3.70°CD8+ cells. As shown in Fig. 6, even in Apaf1−/− thymocytes, cell viability of T3.70°CD8+ cells was lost by peptide stimulation in a dose-dependent manner, and substantial cells were proven dead at 24 h after stimulation with 10 μM peptide. However, Apaf1−/− T3.70°CD8+ thymocytes were more resistant to peptide stimulation than Apaf1+/− T3.70°CD8+ thymocytes at 10 μM of the peptide. These results indicate the possibility that Apaf1-mediated apoptotic pathway may contribute at least partially to PCD pathway of negative selection in a certain condition.

We then investigated the state of caspase 3 activation in Apaf1−/− thymocytes and its relationship with cell viability during negative selection in our in vitro stimulation system. After induction of negative selection by peptide stimulation, we evaluated for dead cells by PI staining and cells positive for active caspase 3 by PhiPhiLuxG2D2 in CD8+ T3.70° thymocytes. PhiPhiLuxG2D2 is a cell-permeable fluorogenic caspase substrate with specificity for caspase 3 (and, possibly, related caspases), and detects caspase activation in apoptotic cells without fixation (26). As shown in Fig. 7A, positive signal for caspase activation was detected in most of Apaf1−/− CD8+T3.70° thymocytes after 10 μM peptide stimulation, and most of them underwent cell death. In Apaf1−/− CD8+ T3.70° thymocytes, cells positive for caspase activation signal remarkably decreased compared with Apaf1+/− CD8+ T3.70° thymocytes. Concomitant with this decreased caspase activity, the percentage of dead cells in Apaf1−/− CD8+ T3.70° thymocytes also decreased. Thus, it is demonstrated that TCR ligation induces caspase activation in an Apaf1-dependent way, further substantiating the possible involvement of Apaf1 in negative selection. However, substantial caspase activation was still observed in Apaf1-deficient cells after peptide stimulation, and cell death was also induced, indicating caspase activation via Apaf1-independent apoptotic pathway also took part in the negative selection. Western blot analysis of caspase activation revealed caspase 3 activation in peptide-stimulated wild-type and Apaf1-deficient thymocytes (Fig. 7B). There was no detectable level of caspase 3 and 6 activation both in wild-type and Apaf1-deficient thymocytes in response to the peptide stimulation, indicating caspase 3 was the dominant caspase activated by the stimulation.

Additionally, to examine the requirement of caspase activity in the negative selection, we also took advantage of a broad spectrum caspase inhibitor, z-VAD-fmk, in this assay. Notably, even in the presence of z-VAD-fmk, although the dead cells remarkably decreased compared with the culture without the inhibitor, peptide stimulation apparently induced cell death of Apaf1−/− thymocytes when caspase 3 activation was completely inhibited. Increasing dose of z-VAD-fmk up to 200 μM did not prevent the cell death (data not shown), indicating that cell death observed in the presence of z-VAD-fmk is caspase independent. However, with relatively weak inhibitory activities of z-VAD-fmk toward other caspases than caspase 3 along with its short half-life, it is not formally excluded that the cell death in the presence of z-VAD-fmk is still caspase dependent. In any case, these data, taken together, indicate that the cell death during the negative selection is caused dominantly by the Apaf1-independent pathway and partially or supplemen tally by Apaf1-mediated pathway in a certain situation; the former pathway may consist of caspase-dependent (z-VAD-inhibitable) one and caspase-independent (z-VAD-uninhibitable) one.

Discussion
Mitochondria-dependent apoptotic pathway, i.e., the apoptotic pathway via mitochondria→cyto c→Apaf1→caspase 9→caspase 3, has been demonstrated in many reports to be critical for development of the body and maintenance of homeostasis of various tissues (16–21). Since PCD plays a critical role in thymocyte selection, we examined the role of Apaf1, a central element in the mitochondria-dependent apoptotic pathway, in the development of thymocytes. Although Apaf1−/− thymocytes show resistance to a
wide range of apoptotic stimuli (19), our results clearly showed that Apaf1-mediated apoptotic pathway is not essential for development of thymocytes (Fig. 1). Similar observations have been reported for the thymocytes of caspase 3- or caspase 9-deficient mice (16–18). Thus, mitochondria-mediated apoptosis appears to be unnecessary for development of thymus. In this study, we first examined the involvement of the Apaf1-dependent apoptotic pathway in thymic negative selection using Apaf1−/− thymocytes in H-Y Tg and SEB-induced deletion system in FTOC, and demonstrated that this pathway is not essential for the PCD during this selection process. However, although our data clearly showed that Apaf1 is unnecessary for the negative selection, it is not excluded that another Apaf1-like molecule(s), which has yet to be identified, is possibly at play in PCD during the negative selection.

Bcl-2 and Bcl-xL are members of the Bcl-2 family with anti-apoptotic function. It has been shown that these molecules exert their antiapoptotic role by inhibiting mitochondrial membrane disruption, thereby working upstream of Apaf1 (31, 32). Thus, when overexpressed, these molecules render the resistance of cells to apoptosis induced by mitochondria-damaging stimuli. Actually, thymocytes of Bcl-2 or Bcl-xL Tg mice show resistance to deletion induced in vivo by anti-CD3 Ab (33, 34). Nevertheless, it has been reported that the negative selection of thymocytes, induced by endogenous superantigen or by H-Y Ag in H-Y Tg model, is normal in these mice (33–35). However, in contrast to these reports, Strasser et al. (36) also reported that Tg expression of bcl-2 Tg mice diminished self Ag-reactive H-Y Tg T cells. Thus, although controversial, antiapoptotic members of Bcl-2 family may play roles during the negative selection of the thymocytes.

In this study, we demonstrated that thymocyte stimulation with relevant antigenic peptide caused mitochondrial alterations, such as dissipation of ΔΨm and cyto c release, although not at a striking degree. We, however, also showed Apaf1 is not necessary for execution of the negative selection. In addition, Kuida et al. (16) demonstrated normal susceptibility to TCR stimulation-induced apoptosis of caspase 3-deficient thymocytes, while Hakem et al. (18) showed normal caspase 3 activation in caspase 9-deficient thymocytes. These results suggest that all the apoptotic events commencing with mitochondrial damage do not play roles during the physiological negative selection process, although partial involvement of the pathway is not excluded. In this context, another view of the function of antiapoptotic Bcl-2 family members should be of note. Strasser et al. (37) proposed a model in which antiapoptotic proteins (such as Bcl-2) keep adaptor proteins (Apaf1 or Apaf1-related molecule(s)) from activating caspases, as observed in the complex formation of CED-4 and CED-9 in Caenorhabditis elegans (38). In this model, adaptor proteins may exert their pro-apoptotic effect when freed from antiapoptotic proteins, and the effect may be independent from mitochondrial alterations.

Ligation of the death receptors, or receptors for TNF family members, such as Fas, TNFR1, etc., has been shown also to induce apoptosis of thymocytes (6). Binding of ligands to these death receptors on thymocytes has been shown to trigger the activation of caspase 8 through the adopter molecule Fas-associated death domain (41, 42). In addition, Smith et al. (43) demonstrated that inhibition of caspase 8 activity by Tg expression of CrmA did not impair the deletion of self-reactive T lymphocytes. These lines of evidence suggest that death receptor-mediated apoptotic pathway is also dispensable for this process, as is the mitochondria-mediated apoptosis.

Caspases are critical mediators and effectors of apoptosis (44, 45). It has been shown that caspase 3 is activated in the thymocytes during apoptosis induced in vitro by dexamethasone, anti-CD3

FIGURE 7. Apaf1-dependent and Apaf1-independent caspase activation and caspase-independent cell death in thymocyte induced by specific antigenic peptide. A, Thymocytes (5 × 104/well) from female H-Y Tg/ Apaf1−/− or H-Y Tg/Apaf1−/− fetal liver-transferred chimera were cultured with 10 μM H-Y peptide for 24 h in the presence or absence of 100 μg/ml z-VAD-fmk. T3.70 CD8+ cells were analyzed for dead cells using PI staining (upper, closed histograms) and caspase 3 activity using PhiPhiLuxG2D2 staining (lower, open histograms). Cells incubated at 4°C were used as a negative control. Values in the histograms indicate the percentages of cells positive for PI or active caspase 3. Representative data from three independent experiments with similar results are shown. B, Thymocytes from Apaf1 wild-type or Apaf1-deficient fetal liver-transferred chimeric mice were stimulated with the antigenic peptide at 10 μM or staurosporine (STS) at 0.5 μM for 24 h. Cells were analyzed for dead cells using PI staining (upper, closed histograms) and caspase 3 activity using PhiPhiLuxG2D2 staining (lower, open histograms). Cells incubated at 4°C were used as a negative control. Values in the histograms indicate the percentages of cells positive for PI or active caspase 3. Representative data from three independent experiments with similar results are shown.
mAb, or specific antigen peptide, and that inhibition of this enzymatic activity by addition of caspase inhibitor z-VAD-fmk prevents cell death (29, 30). Actually, Izquierdo et al. (46) showed that negative selection in vivo of thymocytes triggered by two exogenous Ags, SEB and an antigenic peptide in the F5 TCR Tg model, was specifically inhibited in mice Tg for baculovirus p35 protein that is a broad-range caspase inhibitor. However, the contribution of caspasas in cell death during negative selection is controversial. Contrary to Izquierdo’s report, a recent report by Doerfler et al. (47) showed that caspase inhibition by p35 Tg did not block negative selection induced by antigenic peptide in vitro in OT1 Tg model, and by endogenous Ag in H-Y Tg model. This discrepancy may arise from the strength and/or duration of Ag stimulation, since Izquierdo et al. also reported that the expression of p35 was not able to inhibit thymocyte deletion induced by high Ag concentrations or by chronic Ag treatment, and negative selection by endogenous superantigens. Thus, in the physiological environment in which there is sustained stimulation by endogenous Ags, caspase-dependent pathway is activated to eliminate possibly autoreactive T cells, and the blockage of this pathway may be compensated for by other mechanisms. In agreement with this assumption, we showed that Apaf1-deficient thymocytes bearing H-Y Ag-specific TCRs were completely deleted in vivo in the male chimeric mice (Figs. 4B and 5) while showing more resistance to Ag stimulation-induced cell death in vitro at the highest concentration of the peptide (Figs. 6 and 7A). The physiological relevance of the concentration of antigenic peptide used is unclear, however. In any case, the Apaf1-independent cell death pathway per se is sufficient to complete the negative selection. Actually, a stress-activated protein kinase pathway involving mitogen-activated protein kinase kinase 6→p38 activation, known to induce apoptosis in response to various stress, is reportedly sufficient for providing negative selection signal (48). Thus, this pathway is a strong candidate for this caspase-independent pathway of cell death in negative selection.

In summary, our results demonstrated that Apaf1-dependent apoptotic pathway is not essential to PCD during negative selection. However, this apoptotic pathway may contribute at least partially to the negative selection. Beside this pathway, Apaf1-independent pathways dominantly contribute to cell death in the negative selection. Therefore, we suggest that the process of negative selection is composed of several death pathways and these pathways collaboratively work for the completion of negative selection in thymocytes, presumably compensating each other. Involvement of multipathways in the negative selection is reasonable to avoid autoimmune diseases as a result of a failure in negative selection, because a defect in one pathway can be compensated for by other pathways.

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