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J Immunol 2000; 165:2970-2974; doi: 10.4049/jimmunol.165.6.2970
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Spontaneous Thymocyte Apoptosis Is Regulated by a Mitochondron-Mediated Signaling Pathway

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Most thymocytes that have not successfully rearranged their TCR genes or that express a receptor with subthreshold avidity for self-Ag/MHC enter a default apoptosis pathway, death by neglect. Spontaneous thymocyte apoptosis (STA), at least in part, may mimic this process in vitro. However, the molecular mechanism(s) by which thymocytes undergo this spontaneous apoptosis remains unknown. Here, we report that caspase-1 and caspase-3 are activated during STA, but these caspses are dispensable for this apoptotic process. The inhibition of STA by a pan-caspase inhibitor, zVAD, suggests that multiple caspase pathways exist. Importantly, the early release of cytochrome c from mitochondria closely correlates with the degradation of Bcl-2 and Bcl-xL, and a decrease in the ratios of Bcl-2 and Bcl-xL to Bax during STA. These findings suggest that the degradation of Bcl-2 and Bcl-xL may favor Bax to induce cytochrome c release from mitochondria, which subsequently activates downstream caspses in STA. Our data provide the first biochemical insight into the molecular mechanism of STA. The Journal of Immunology, 2000, 165: 2970–2974.

Apoptosis is an important mechanism of maintaining homeostasis during development (1, 2). In the thymus, most thymocytes that have not successfully rearranged their TCR genes or express a receptor with subthreshold avidity for self-Ag/MHC enter a default apoptosis pathway, called death by neglect (3). The molecular mechanism(s) by which thymocytes die by neglect is unknown. Spontaneous thymocyte apoptosis (STA)1 may mimic in part this process in vitro.

The key apoptosis effectors in mammals are a family of cysteine-containing, aspartate-specific proteases called caspses (4, 5). Caspases exist as dormant proenzymes in healthy cells and are activated through proteolysis. Once activated, caspases cleave cellular substrates, leading to morphological hallmarks of apoptosis, including DNA fragmentation and condensation of cellular organelles (2, 4). However, the mechanism of activation of the caspase cascades is not completely understood. Two major pathways have been identified: one is the Fas-mediated recruitment of Fas-associated death domain protein, which leads to the autoproteolytic activation of caspase-8 (2), and the other is a recently discovered mechanism involving the release of cytochrome c from mitochondria, which also involves caspase activation (6, 7). Recent evidence suggests that these two pathways may converge at caspase-8 (8). It has been shown that Bcl-2 and Bcl-xL prevent apoptosis by inhibiting mitochondrial cytochrome c release (6, 9).

Consistent with this idea, thymocytes from Bcl-2 transgenic mice live longer in vitro than those from wild-type mice (10), indicating a role for the mitochondrion or mitochondrion-dependent events in STA.

To test whether mitochondrion-dependent signaling events mediate STA, we examined caspase activation, changes in mitochondrial membrane potential (ΔΨm), cytochrome c release from the mitochondria, and the role of Bcl-2 family proteins in STA. We found that cytochrome c release from mitochondria is an early event in STA, whereas a reduction in ΔΨm appears to be a relatively late event. Our data suggest that a decreased ratio of Bcl-xL or Bcl-2 to Bax might induce cytochrome c release from mitochondria and subsequently activate downstream caspses in STA.

Materials and Methods

Mice and reagents

BALB/c mice of 6–10 wk of age were purchased from the National Cancer Institute (Bethesda, MD). B6Sn.C3H-Fas ligand (FasL) (gld) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). These mice were maintained in the animal facility at Rush-Presbyterian-St. Luke’s Medical Center (Chicago, IL). The following reagents were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): rabbit polyclonal Abs against mouse Bcl-xL (L-19), Bcl-2 (C-2), caspase-1 (M-19), caspase-8 (T-16), Bax (I-19), cytochrome c (H-104), HRP-coupled goat anti-rabbit IgG or rabbit anti-mouse IgG, and mAb against mouse caspase-9 (C-8). Anti-actin mAb (A40) and propidium iodide (PI) were obtained from Sigma (St. Louis, MO). An anti-caspase-3 (CPP32) anti-serum was a gift from Dr. R.-P. Sékaly (University of Montreal, Montreal, Canada). 3,3′-Diisohexyloxacarbocyanine iodide (DiOC6(3)) was purchased from Molecular Probes (Eugene, OR). Caspase inhibitors YVAD-chloromethylketone, DEVD-fluoromethylketone (FMK), LEHD-FMK, and zVAD-FMK were obtained from Cedarslane Laboratories (San Diego, CA), and p38 mitogen-activated protein kinase (p38 MAPK) inhibitor SB203580 was a gift from Dr. P. Young (SmithKline Beecham Pharmaceuticals, King of Prussia, PA).

Detection of STA

Freshly isolated thymocytes (2 × 106/ml) in 24-well plates were cultured in RPMI 1640 medium containing 10% heat-inactivated FCS, 10 mM HEPES, 0.1 mg/ml streptomycin, 100 U/ml penicillin, 0.05 mM 2-ME, and 2 mM glutamine (all from Life Technologies, Grand Island, NY) for different time periods as indicated. For the detection of apoptotic cells, thymocytes were stained with PI. At each time point, cells were harvested, washed with PBS (0.5% glucose), and fixed in cold 70% ethanol overnight. Fixed cells were pelleted to remove ethanol, stained with PI (final concentration, 50 μg/ml) for 30 min at room temperature, and determined using a FACScan with CellQuest software (Becton Dickinson, Mountain View, CA).
Measurement of $\Delta \psi_m$ by flow cytometry

The $\Delta \psi_m$ results from the asymmetric distribution of protons across the inner mitochondrial membrane, giving rise to a chemical (pH) and electric gradient (9, 11, 12). Cells that undergo apoptosis manifest a reduction in the incorporation of $\Delta \psi_m$-sensitive dyes, indicating a disruption of $\Delta \psi_m$. For DiOC$_6$(3) staining, $10^6$ thymocytes were incubated with DiOC$_6$(3) (at a final concentration of 40 nM in PBS) for 20 min at 37°C and analyzed immediately using a FACSscan.

Preparation of cell lysates and cytosolic extracts

Thymocytes at each time point of culture were collected and lysed in ice-cold lysis buffer containing 1% Triton X-100, 10 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EGTA, 50 mM β-glycerophosphate, 2 mM Na$_2$VO$_4$, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Alternatively, cytosolic extracts were isolated as previously described (13). Cell homogenates were spun at 14,000 $\times$ g for 15 min, and supernatants were collected and stored at $\sim$80°C until use.

Electrophoresis and immunoblotting

Protein concentrations from cell lysates and cytosolic extracts were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Equal amounts (30 μg) of proteins from either cell lysates or cytosolic extracts was loaded onto each lane of 12 or 15% SDS-PAGE. The proteins were electrophoretically separated and then transferred to nitrocellulose membranes (Amersham, Piscataway, NJ). Anti-caspase 3 and anti-actin Abs were diluted at 1/500, and the other Abs were diluted at 1/1000. The membranes were incubated with HRP-coupled goat anti-rabbit IgG or rabbit anti-mouse IgG at 1/5000 or 1/3000 for 2 h at room temperature. To confirm the equal loading of proteins, the membranes were stripped and reprobed with anti-actin mAb. The specific proteins were identified using the ECL system (Amersham).

Statistical analysis

A two-way ANOVA and $t$ tests were performed to determine whether differences in STA after pretreatment with various inhibitors were significant (StatView software, Abacus Concepts, San Francisco, CA).

Results and Discussion

Activation of other caspases, but not caspase-1 and caspase-3, mediates STA

Caspases play key roles in apoptosis (1, 2, 4, 5). Thymocytes undergo spontaneous apoptosis when cultured in vitro. As shown in Fig. 1A, STA began between 8–24 h of culture and increased with time. At 72–96 h of culture, $\sim$70–90% of thymocytes was dead as determined by PI staining. To confirm whether pro-caspase-1 and pro-caspase-3 were activated during STA, the cleavages of these pro-caspases were detected by immunoblotting using anti-caspase-1 and anti-caspase-3 Abs, respectively. Indeed, the activation of pro-caspase-1 and pro-caspase-3 was indicated by the disappearance of pro-enzyme forms and the appearance of active fragments (Fig. 1B).

![Figure 1](http://www.jimmunol.org/)
To further test the roles of caspases in STA, several caspase inhibitors, such as YVAD for caspase-1, DEVD for caspase-3, and zVAD for pan-caspases, were used. YVAD and DEVD failed to inhibit spontaneous apoptosis, whereas the pan-caspase inhibitor zVAD at concentrations of 50–70 μM significantly suppressed STA, with optimal inhibition at 60 μM (Fig. 1C). This observation was consistent with an early study (14). The inhibition of STA, however, could not be simply ascribed to the suppression of caspase-3 activation as described in that report, because the pan-caspase inhibitor zVAD was used (14). Notably, high concentrations of YVAD and DEVD (≥50 μM) and zVAD (≥80 μM) proved to be toxic to thymocytes, because STA was accelerated at this concentration of inhibitors (Fig. 1C). The failure of the inhibition of caspase-1 and caspase-3 by YVAD and DEVD was not due to the inactivation of these inhibitors in the culture system, as pretreatment of activated splenic T cells with YVAD and DEVD significantly inhibited activation-induced cell death (AICD; Fig. 1D) (15). These data suggest that STA is independent of the activation of caspase-1 and caspase-3 and may be mediated by an alternative caspase pathway(s). Interestingly, caspase-3 activation was observed in the absence of apoptosis during T cell activation (16), indicating that caspase-3 activation can occur independently of apoptosis in T cells. Taken together, these two pieces of evidence suggest that the activation of caspase-3 may be required for some, but not all, types of apoptosis. Because we previously showed that the inhibition of p38 MAPK could suppress AICD (15), we examined whether p38 MAPK played a role in STA. Pretreatment of thymocytes with different doses of the p38 MAPK-specific inhibitor, SB203580, failed to inhibit STA (Fig. 1C), suggesting that the signaling pathways required for AICD and STA are different.

Caspase-8 is the most upstream caspase identified in the Fas-mediated signaling pathway, and its activation can trigger the mitochondrial-mediated death pathway by cleaving Bcl-2-interacting death protein and subsequently inducing the release of cytochrome c from mitochondria (6, 8, 17). It has been shown that mouse thymocytes express high levels of Fas (18, 19). To confirm that STA was not mediated by Fas-FasL interaction, the time kinetics of Fas and FasL expression during STA were determined using flow cytometry. Fas expression was enhanced with increasing culture times, and by the end of the culture (72 h), it was >2-fold higher than that detected in freshly isolated thymocytes (Fig. 2A). The nonspecific staining was excluded by FITC- and PE-conjugated isotype-matched control IgG. One representative of three independent experiments is shown. B, Thymocytes from gld mice were cultured for 0, 24, 48, 72, and 96 h. STA was determined at each time point. C, Thymocytes cultured for different time periods or stimulated with immobilized anti-Fas mAb (10 μg/ml) for 16 h were collected and lysed. Cell lysates were separated on SDS-PAGE gel, transferred, and probed with anti-caspase-8 Ab. The membranes were stripped and reprobed with anti-actin mAb. One representative of three independent experiments is shown.

Cytosolic cytochrome c release from mitochondria precedes a reduction in Δψm.

The mitochondrion is a pivotal decision center that controls life and death by releasing death-promoting factors into the cytosol (6). One of these factors is cytochrome c, a protein that normally shuttles electrons between protein complexes in the inner mitochondrial membrane. Once released, cytochrome c helps to activate caspases such as apoptotic protease activating factor-1 (Apaf-1) and caspase-9 (6, 17). Two competing models have been proposed to explain how cytochrome c is released from the mitochondria. In the first model, the permeability transition pore opens, allowing water and solutes to enter. The mitochondrion swells, its outer membrane ruptures, and the mitochondrial proteins, including cytochrome c, escape. The second model suggests the formation of a channel that is large enough to allow cytochrome c to pass through (6, 21).

To examine the sequence of events that link mitochondrial cytochrome c release to other molecular events in STA, cytochrome c release, Δψm, and caspase-9 activity were monitored simultaneously during STA. Cytosolic extracts were prepared at various times from thymocytes in culture under conditions that keep mitochondria intact. Cytosolic release of cytochrome c protein was assessed by immunoblotting. Cytosolic extracts from freshly isolated thymocytes contained very little cytochrome c. At 8–16 h of culture, cytochrome c was maximally released from mitochondria; cytosolic cytochrome c gradually declined thereafter and disappeared after 24 h of the culture (Fig. 3A). It was noteworthy that cytochrome c release preceded maximal spontaneous apoptosis, suggesting that the release of cytochrome c from mitochondria is an early event in STA.

The reduction in Δψm that accompanies early apoptosis in many experimental systems is believed to be mediated by the opening of
the mitochondrial permeability transition pore, a multiprotein complex (6, 21). To determine whether a reduction in Δψm was an early or a late event in STA, thymocytes in the culture were collected at different time points, stained with the fluorochrome DiOC6(3), and assayed by flow cytometry. As shown in Fig. 3B, a reduction in Δψm only occurred at 48 h of culture, which correlated with evident STA and the activation of caspases. Our observations suggest that a reduction in Δψm is a late event in STA, which favors the second model for the release of cytochrome c from mitochondria. Moreover, zVAD has been shown to effectively block the reduction in Δψm induced by apoptosis-inducing agents, but failed to block the release of cytochrome c (6). This finding suggests that a reduction in Δψm may be a consequence of caspase activity rather than the effector mechanism driving cytochrome c efflux (13). In agreement with this idea, treatment of isolated mitochondria with the protonophore and uncoupler m-chlorophenylhydrazine led to a rapid decrease in Δψm, yet failed to elicit cytochrome c release and apoptosis (22). Thus, the release of cytochrome c from mitochondria to the cytosol may not require a mitochondrial transmembrane depolarization in STA. Our observations together with others (13) suggest that the disruption of Δψm may be critical for apoptosis in some cases, but it is not a universal step in the apoptotic process.

Cytochrome c activates caspases by binding to Apaf-1, inducing the association of Apaf-1 with pro-caspase-9, thereby triggering caspase-9 activation and initiating the proteolytic cascade that culminates in apoptosis (9, 17). We therefore examined the activation of caspase-9 by monitoring the time course of the cleavage of pro-caspase-9 in STA. Similar to the activation of caspase-1 and caspase-3, the cleavage of pro-caspase-9 and appearance of p20 fragment occurred at 24 h of culture, and became evident at 48 h (Fig. 3C), which correlated with the peak of STA (Fig. 1B). Moreover, a caspase-9-specific inhibitor, LEHD, significantly suppressed STA, with the inhibition level comparable to that with zVAD (Fig. 3D). This observation suggests that caspase-9 is crucial for STA. Because we have shown that caspase-1 and caspase-3 are not required for STA and that caspase-8 is not activated during STA, the inhibition of STA by the pan-caspase inhibitor zVAD must be due to the suppression of an alternative novel caspase pathway(s).

Degradation of Bcl-xL and appearance of Bcl-xS correlate with the kinetics of STA

It has been shown that the balance between the life- and death-promoting pathway is controlled by the amounts of Bcl-2-Bax or Bcl-xL-Bax heterodimers present. Life is promoted when the homodimeric forms of Bcl-2:Bcl-2 or Bcl-xL-Bcl-xL are in excess, whereas death is promoted when Bax-Bax homodimers are present (23). Bax has also been shown to be able to induce mitochondrial cytochrome c release, and this release can be inhibited by Bcl-xL (24). To investigate whether a decrease in the ratio of Bcl-2 or Bcl-xL to Bax was associated with STA, thymocyte lysates were blotted with anti-Bcl-2, anti-Bcl-xL, and anti-Bax Abs, respectively. Bcl-2 and Bcl-xL expression was decreased with the culture time, whereas the expression of Bax protein remained unchanged during entire course of culture (Fig. 4B). Degradation of both Bcl-2 and Bcl-xL occurred after 8 h of culture, which correlated with the release of cytochrome c from mitochondria. Interestingly, the degradation of Bcl-xL was associated with the appearance of Bcl-xS (Fig. 4A), which correlated closely with the kinetics of

FIGURE 4. Imbalance of the ratio of Bcl-2 and Bcl-xL to Bax may promote STA. Thymocytes cultured for different times were lysed, separated by 15% SDS-PAGE gels, and blotted with anti-Bcl-2, anti-Bcl-xL, and anti-Bax Abs as indicated. Data shown are from one of three independent experiments.
STA. It has been shown that Bcl-2 and Bcl-x<sub>L</sub> can inhibit cytochrome c release from mitochondria (6, 9, 24); therefore, it is likely that the degradation of Bcl-2 and Bcl-x<sub>L</sub> and the concurrent appearance of Bcl-x<sub>L</sub> play a role in STA by regulating mitochondrial cytochrome c release.

The signaling mechanism for thymocyte death by neglect is unknown. It is believed that this special type of thymocyte death is TCR independent, which is similar to STA. Recently, it has been reported that thymic glucocorticoids may regulate STA by setting the thresholds for thymic selection (25, 26). Thymic epithelial cells may induce thymoma cell death by neglect in an in vitro system (27), but it is not clear whether thymic epithelial cells in this report influence thymocyte death via glucocorticoid. It remains to be further elucidated how steroid hormone and cell-cell interaction affect STA or death induced by neglect.

Although the lot of FCS may influence cell viability, we found that the variation in STA caused by FCS from different lots (Sigma and HyClone (Logan, UT)) was ~5–10%. This variation does not affect the biochemical changes in STA, e.g., caspase activation, expression of Bcl-2, Bcl-x<sub>L</sub> and Bax, and cytochrome c release (data not shown).

In summary, our results indicate that caspases are indispensable for STA, but caspase-1 and caspase-3 are not involved in this apoptotic process. The early release of cytochrome c from mitochondria, the degradation of Bcl-x<sub>L</sub> concomitantly with the appearance of Bcl-x<sub>L</sub>, and the imbalance of the ratio of Bcl-2 to Bcl-x<sub>L</sub> to Bax suggest that these mitochondrion-mediated events may play crucial roles in STA. Degradation of Bcl-x<sub>L</sub> and Bcl-2 results in a relative increase in the amount of Bax that is responsible for the induction of cytochrome c release from mitochondria and subsequent activation of downstream caspasess (Fig. 5). Our findings provide the first biochemical insight into STA, a possible important regulatory process in the maintenance of thymocyte homeostasis.

**Acknowledgments**

We thank Drs. R.-P. Sekaly and P. Young for providing polyclonal Ab against CPP32 and SB203580, Dr. T. Bardos for his technical assistance, and Sonja Velins for her valuable assistance with the preparation of this manuscript.

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