Proteasome Activation Occurs at an Early, Premitochondrial Step of Thymocyte Apoptosis

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Proteasome Activation Occurs at an Early, Premitochondrial Step of Thymocyte Apoptosis

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Proteasomes and mitochondrial membrane changes are involved in thymocyte apoptosis. The hierarchical relationship between protease activation and mitochondrial alterations has been elusive. Here we show that inhibition of proteasomes by two specific agents, lactacystin or MG132, prevents all manifestations of thymocyte apoptosis induced by the glucocorticoid receptor agonist dexamethasone or by the topoisomerase II inhibitor etoposide. Lactacystin and MG132 prevent the early disruption of the mitochondrial transmembrane potential ($\Delta \Psi_m$), which precedes caspase activation, exposure of phosphatidylserine, and nuclear DNA fragmentation. In contrast, stabilization of the $\Delta \Psi_m$ using the permeability transition pore inhibitor bongkrekic acid or inhibition of caspases by $N$-benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone does not prevent the activation of proteasomes, as determined with the fluorogenic substrate $N$-succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-7-amido-4-methylcoumarin. Thus, proteasome activation occurs upstream from mitochondrial changes and caspase activation. Whereas the proteasome-specific agents lactacystin and MG132 truly maintain thymocyte viability, a number of protease inhibitors that inhibit nuclear DNA fragmentation (acetyl-Asp-Glu-Val-Asp-fluoromethylketone; $N$-Boc-Asp(OMe)-fluoromethylketone; $N$-tosyl-L-Phexachloromethylketone) do not prevent the cytolytic induced by DEX or etoposide. These latter agents fail to interfere with the preapoptotic $\Delta \Psi_m$ disruption. Altogether, our data indicate that different proteases may be involved in the pre- or postmitochondrial phase of apoptosis. Only those protease inhibitors that interrupt the apoptotic process at the premitochondrial stage can actually preserve cell viability.


Apoptosis may be defined as a form of cell death in which the activation of catabolic hydrolyses (proteases and nuclease), within a near-to-intact plasma membrane, contributes to the acquisition of a stereotyped pattern of biochemical and morphologic alterations (1, 2). Overwhelming evidence suggests the involvement of specific cysteine proteases cleaving after aspartic acid (caspases), which catalyze a highly selective pattern of protein degradation, in apoptosis (3–7). Inhibitors acting on a wide range of caspases such as the Baculovirus protein $\beta$5 (8–10), $N$-benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone ($\beta$-VAD.fmk)3 (11–19), its truncated analogue Boc-Asp(OMe)-fluoromethylketone (B-D.fmk) (16), or acetyl-Asp-Glu-Val-Asp-fluoromethylketone (Ac-DEVD.fmk) (20) inhibit the acquisition of apoptotic morphology and endonuclease activation in most if not all experimental models. In addition to caspases, different serine proteases, calpains, and proteasomes have been implicated in the apoptotic process (7). Thus, proteasome inhibitors such as lactacystin and MG132 protect thymocytes, T cell hybridomas, and PC12 cells against a number of different apoptosis inducers (glucocorticoids, etoposide, irradiation, anti-CD3, ligation of CD95, withdrawal of nerve cell growth factor) (21–24). Proteasomes are also involved in the developmentally programmed cell death of the intersegmental muscle of the insect Manduca sexta (25, 26), suggesting that they may play a major role throughout evolution. However, they appear to be less common effectors of the apoptotic pathway than caspases, given that inhibitors of proteasomes are ineffective in some models of apoptosis (27) and actually can induce apoptosis in proliferating cell lines (23, 28–31).

Apoptosis is also characterized by changes in mitochondrial membrane function. These changes affect the inner membrane, causing a perturbation of the transmembrane potential ($\Delta \Psi_m$), which at least in some cases is preceded by an increase in the matrix volume (32, 33). They also affect the outer membrane through which apoptogenic factors leak into the cytosol (32–36). It has been proposed that the apoptotic process can be subdivided into three phases: 1) a heterogeneous premitochondrial initiation phase during which receptor-mediated stimuli or damage pathways act on the cell; 2) a common mitochondrial effector phase, during which mitochondrial membrane alterations occur; and 3) a postmitochondrial degradation stage, during which apoptogenic factors released from the mitochondrial intermembrane space activate proteases, mainly caspases, and nucleases (32, 37). The mechanisms linking caspase activation and mitochondrial changes appear complex. Upstream caspases activated by CD95 cross-linking during the premitochondrial initiation stage can induce an increase in mitochondrial membrane permeability, both in cells and in isolated

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2 Abbreviations used in this paper: $\beta$-VAD.fmk, $N$-benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Ac-DEVD.fmk, acetyl-Asp-Glu-Val-Asp-fluoromethylketone; B-D.fmk, Boc-Asp(OMe)-fluoromethylketone; $\Delta \Psi_m$, mitochondrial transmembrane potential; DEX, dexamethasone; Eth, ethidium; $\Delta \psi$, mitochondrial transmembrane potential; PIC12 cells against a number of different apoptosis inducers (glucocorticoids, etoposide, irradiation, anti-CD3, ligation of CD95, withdrawal of nerve cell growth factor) (21–24). Proteasomes are also involved in the developmentally programmed cell death of the intersegmental muscle of the insect Manduca sexta (25, 26), suggesting that they may play a major role throughout evolution. However, they appear to be less common effectors of the apoptotic pathway than caspases, given that inhibitors of proteasomes are ineffective in some models of apoptosis (27) and actually can induce apoptosis in proliferating cell lines (23, 28–31).

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mitochondria (38). In addition, at least two different apoptogenic proteins released from mitochondria, cytochrome c and AIF, can activate downstream caspases that participate in the postmitochondrial stage of apoptosis (34–36, 38).

No information is available on the relationship between non-caspase proteases and mitochondrial function. We therefore decided to study the temporal and functional relationship between proteasome activation and mitochondrial changes in a model of thymocyte apoptosis, namely apoptosis induced by glucocorticoids or etoposide. Our results indicate that proteasomes act upstream of mitochondria, whereas proteases inhibited by Ac-DEVD.fmk, B-D.fmk), or N-tosyl-l-phenylalanine chloromethylketone (TPCK) act downstream of the ΔΨm disruption. In the models that we have studied, only those protease inhibitors that act upstream of mitochondria can actually preserve cell viability.

Materials and Methods
Inhibition and induction of apoptosis

Thymocytes from female 4- to 6-wk-old BALB/c mice were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, and antibiotics. Cells (1–5 × 10⁶/ml) were cultured in a humidified atmosphere containing 5% CO₂ at 37°C during the indicated interval (5 to 12 h), alone or with combinations of the following reagents: dexamethasone (DEX; 1 μM, Sigma, St. Louis, MO); etoposide (VP-16, 10 μM, Sigma); MG132 (CBZ-leucyl-leucyl-leucinal, 30 μM, Peptides International, Louisville, KY); lactacystin (30 μM, purchased from Dr. M. R. Corey, Harvard University, Cambridge, MA); N-acetyl-l-leucinyl-l-leucinal-l-norleucinal (LNNL, 30 μM, also called “calpain inhibitor I; Bachem, Basel, Switzerland); N-tosyl-l-phenylalanine chloromethylketone (TPCK, 10 μM, Sigma); N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAL-OMe, 50 μM, Enzyme Systems, Dublin, CA); Boc-Asp(OMe)-fluoromethylketone (B-D.fmk, 50 μM, Enzyme Systems); acetyl-Asp-Glu-Val-Asp-fluoromethylketone (Ac-DEVD.fmk, 50 μM, Enzyme Systems); and/or bongkrekic acid (BA, 50 μM, provided by Dr. Duine, Delft University, Delft, The Netherlands). Preliminary experiments were performed with variable doses of these inhibitors (1 to 200 μM), and results are shown for optimal nontoxic concentrations of each agent.

![FIGURE 1. Stimulation of proteasome activity by etoposide or DEX. Thymocytes were cultured for 5 h in the presence of the indicated agents (gray areas), followed by addition of the proteasome substrate N-succinyl-t-leucyl-t-leucyl-t-tyrosine-7-amido-4-methylcoumarin and the determination of 7-amino-4-methylcoumarin fluorescence. This experiment was repeated twice yielding similar results.](http://www.jimmunol.org/)

![FIGURE 2. Effects of three proteasome inhibitors (MG132, lactacystin, and LNNL) on thymocyte apoptosis induced by DEX or etoposide. Cells were cultured for 5 h in the presence of the indicated combination of agents, followed by staining with DiOC₆(3) and HE (A) or FITC-annexin V and EthBr (B), as described in Materials and Methods. Numbers refer to percentage of cells found in each quadrant. In addition, aliquots of cells were fixed and permeabilized with ethanol and stained with PI to determine the frequency of hypoploid nuclei (numbers in black circles in A). Results are representative of three independent experiments.](http://www.jimmunol.org/)
Determination of apoptosis-associated parameters

In accord with published protocols (39–41), the following fluorochromes were used to determine different apoptosis-associated changes: 3,3′-di-hexyloxycarbocyanine iodide (DiOC6(3); 20 nM, 15 min, 37°C) for ΔΨm quantification; hydroethidine (HE, 4 μM, 15 min, 37°C) for the determination of superoxide anion generation causing oxidation of HE to ethidium (Eth); annexin V-FITC (1 μg/ml, 10 min, 4°C, Nexins Research, Hoeven, The Netherlands) for determination of phosphatidylserine (PS) exposure on the outer plasma membrane; and finally ethidium bromide (EthBr; 200 ng/ml, 5 min, room temperature) or propidium iodide (PI, 2 μg/ml, 5 min, 37°C), which both are vital dyes. These dyes were used alone (PI) or in combination (DiOC6(3) + HE; annexin V-FITC + EthBr). Cytomorphometry was performed on a Coulter Elite II analyzer. Fluorescence was registered for all cells (large and apoptotic), while excluding debris, in FL1 (DiOC6(3), annexin V-FITC) or FL3 (HE, EthBr, PI). The frequency of subdiploid cells was determined by PI staining of ethanol-permeabilized cells (41).

Determination of proteasome activity

Proteasome activity was determined by means of the cell-permeable fluorescent generic substrate N-succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-7-amido-4-methylcoumarin (Bachem), following published protocols (42). Cells (4 × 10^5 in 200 μl of PBS, pH 7.4) were incubated during 30 min at 37°C with this substrate (100 μM), and the 7-amino-4-methylcoumarin fluorescence generated by its cleavage was measured in a Kontron SFM 25 spectrofluorometer (Kontron AG, Zurich, Switzerland). The excitation wavelength was set at 380 nm, and the emission wavelength was set at 460 nM. Background values of nonstimulated cells were not reduced by the proteasome inhibitors MG132 (30 μM) or lactacystin (30 μM) and thus were subtracted from the experimental values.

Results and Discussion

Inhibition of proteasomes prevents thymocyte apoptosis at the premitochondrial level

The glucocorticoid receptor agonist DEX and the topoisomerase type II inhibitor etoposide both induce apoptosis in thymocytes. Simultaneously, they cause an increase in proteasome activity, as determined by means of the fluorescent substrate N-succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-7-amido-4-methylcoumarin (Fig. 1). Several different proteasome inhibitors, which have been used at nontoxic doses, reduce this enzymatic activity. This applies to the specific proteasome inhibitors MG132 and lactacystin (21), as well as to LLnL, an inhibitor of proteasomes and calpain (21, 42) (Fig. 1). We have evaluated the effect of these inhibitors on several apoptosis-associated parameters quantified by cytometry (Fig. 2). The mitochondrial transmembrane potential (ΔΨm) was measured using the potential-sensitive cationic lipophilic dye DiOC6(3), which incorporates into the mitochondrial matrix as a function of the Nernst equation, correlating with the ΔΨm (43) (Fig. 2A). The overproduction of superoxide anion by the uncoupled respiratory chain was quantified by the oxidative conversion of nonfluorescent HE into fluorescent Eth (Fig. 3A). Moreover, we measured, among viable (i.e., EthBr-excluding) cells, the apoptosis-associated exposure of PS on the outer leaflet of the plasma membrane using the PS-specific protein annexin V conjugated to FITC (Fig. 2B). The endonuclease-driven loss of nuclear DNA leading to hypoploidy was measured with the DNA-intercalating dye PI, after permeabilization of cells with ethanol. MG132, lactacystin, and LLnL inhibit the dissipation of the ΔΨm induced by DEX and etoposide (Fig. 2A), in addition to suppressing the generation of superoxide anion (Fig. 2A), the loss of nuclear DNA leading to hypoploidy (numbers in black circles in Fig. 2A), and the exposure of PS (Fig. 2B). The cytoprotective effect of these agents correlates with the inhibition of proteasome enzymatic activity (Figs. 1 and 2). Collectively, these results indicate that proteasome inhibition interrupts the apoptotic process at an early step, upstream of mitochondria.

Proteasome activation occurs upstream of ΔΨm dissipation and caspase activation

We have used BA, a ligand of the adenine nucleotide translocator and an inhibitor of the mitochondrial megachannel (also called permeability transition pore) (40, 44–46), and Z-VAD.fmk, a broad spectrum inhibitor of caspases (11–19), to evaluate the impact of the megachannel and caspases on proteasome function. As shown in Figure 3A, neither of these two agents prevents the activation of proteasomes induced by etoposide or DEX. In parallel control experiments, BA stabilizes the ΔΨm (measured by means of the potential-sensitive dye DiOC6(3)) of thymocytes treated with DEX or etoposide (40, 46) (Fig. 3B). Moreover, it prevents the postmitochondrial manifestations of apoptosis including superoxide anion generation (measured by the oxidative conversion of nonfluorescent HE into fluorescent Eth) (Fig. 3B), DNA fragmentation (measured by PI staining of ethanol-permeabilized cells) (Fig. 3C), PS exposure (measured by means of annexin V-FITC conjugate) (Fig. 3C), and maintains cell viability (measured with EthBr) (Fig. 3C) (40), thus confirming that opening of the megachannel is a critical event of the apoptotic process. Z-VAD.fmk blocks most of the apoptosis-associated changes (complete ΔΨm disruption, superoxide anion generation, PS exposure, nuclear apoptosis) (Fig. 3, B and C), with the notable exception of the initial ΔΨm dissipation (19) (Fig. 3B). Thus, both BA and Z-VAD.fmk interrupt the apoptotic process downstream of proteasome activation. These data support the idea that proteasome activation occurs upstream of and independently from ΔΨm dissipation and caspase activation.

FIGURE 3. Effect of BA and the caspase inhibitor Z-VAD.fmk on proteasome activation and apoptosis. Thymocytes were cultured during 5 h in the presence of the indicated agent, followed by determination of proteasome activity (A), as in Figure 1, or the indicated apoptosis-associated parameters (B, C), as in Figure 2. Circles in C indicate the percentage of hypoploid cells.
Among different protease inhibitors, proteasome inhibitors have the selective capacity of maintaining cell viability.

In addition to proteasome inhibitors, a number of protease inhibitors have been reported to prevent thymocyte apoptosis (7, 11, 16, 19). We therefore have evaluated the effect of different protease inhibitors not acting on proteasomes on several parameters of thymocyte apoptosis. Two inhibitors of caspases, B-D.fmk and Ac-DEVD.fmk, which do not inhibit proteasomes (Refs. 21 and 22, Fig. 3A, and data not shown) largely prevent the PI-detectable loss of nuclear DNA (Fig. 4B) and concomitantly prevent oligonucleosomal DNA fragmentation (Ref. 16 and data not shown). Similarly, the chymotryptic inhibitor TPCK, which does not inhibit caspases (47), prevents DNA fragmentation in this model (Fig. 4B). However, these inhibitors are relatively inefficient in preventing the initial step of \( \Delta \Psi_m \) dissipation (Fig. 4A), as has been observed for Z-VAD.fmk (19) (Fig. 3B). Thus, in contrast to proteasome inhibitors (see above), B-D.fmk, Ac-DEVD.fmk, and TPCK act on a postmitochondrial rather than a premitochondrial step of the apoptotic process. On prolonged culture of thymocytes (12 h), B-D.fmk, Ac-DEVD.fmk, and TPCK fail to maintain the viability of cells, as assessed with PI. In the same conditions, however, the proteasome inhibitors MG132 and lactacystin do maintain cell viability (Fig. 5). These results support the contention that only protease inhibitors capable of interrupting the process of apoptosis at the premitochondrial stage (lactacystin, MG132) but not those acting at the postmitochondrial stage (B-D.fmk, Ac-DEVD.fmk, TPCK) are truly cytoprotective.

Concluding remarks

In the models of apoptosis studied herein, inhibition of proteasomes interrupts the process at an early, premitochondrial step. Since proteasomes and mitochondria are usually not found in close association (48), it is unlikely that proteasomes would have a direct effect on mitochondria. At present, it is not known which is/are the critical protein(s) that must be ubiquinated and then degraded by the proteasome to facilitate apoptosis induction. However, it appears plausible to assume that the proteasome participates in one or several signaling pathways during the premitochondrial initiation phase of apoptosis. In accord with this speculation, we found that inhibition of the mitochondrial megachannel and inhibition of caspase activation do not interfere with the activation of proteasomes, whereas MG132 and lactacystin, two specific inhibitors of proteasomes, prevent the \( \Delta \Psi_m \) dissipation induced by etoposide and DEX (Figs. 2 and 3A). Concomitantly, MG132 and lactacystin inhibit all extramitochondrial manifestations of apoptosis including nuclear DNA fragmentation, plasma membrane PS exposure, and cytolysis (Figs. 2 and 3). In strict contrast with these proteasome inhibitors, caspase inhibitors (B-D.fmk, Ac-DEVD.fmk) or...
the chymotryptic inhibitor TPCK fail to prevent the $\Delta\Psi_m$ dissipation (Fig. 4A) and later cytolysis (Fig. 5B), although they do prevent DNA fragmentation (Fig. 4B). These latter agents thus act at a postmitochondrial step and ultimately are incapable of preventing lytic cell death.

The data contained in this work suggest that the manifestation of the mitochondrial changes have a higher predictive value for cell death than the activation of catabolic enzymes including caspases and nucleases. Indeed, in a number of models of apoptosis, the broad spectrum caspase inhibitor Z-VAD.fmk can prevent caspase and consequent nuclease activation yet fails to prevent the disruption of the $\Delta\Psi_m$ and cytolysis. This applies to cell death induced by overexpression of Bax (17), opening of the mitochondrial megachannel by protoporphyrin IX (45), ligation of the glucocorticoid receptor, or topoisomerase II inhibition (19). Moreover, cross-linking of CD45 or CD99 can cause a type of thymocyte death that is preceded by $\Delta\Psi_m$ disruption but does not involve the activation of caspases and nucleases (49, 50). Conversely, activation of caspases can occur without cell death, as this has been demonstrated for activated T cells (51), T cell hybridoma cells transfected with ALG-2 (52), and CD95-stimulated Jurkat cells overexpressing Bcl-XL (53). Altogether these findings support the idea that, at least in some models, mitochondrial changes are more important in determining cell death than caspase activation. It remains to be clarified, however, which changes in cellular physiology finally account for caspase-independent cytolysis. On theoretical grounds, the mitochondrial perturbations occurring during apoptosis should provoke an insufficiency in energy metabolism, ion homeostasis, and/or redox balance that altogether compromise plasma membrane integrity. However, the exact mechanisms linking mitochondrial dysfunction and cell lysis await further characterization.

In conclusion, our data support a scenario in which proteasomes act upstream of mitochondria to regulate cell death, whereas some caspases act downstream of mitochondria to participate in the acquisition of the apoptotic phenotype, beyond the point of no return leading to cell death.

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References

PROTEASOMES IN THYMOCYTE APOPTOSIS


