

Differential Regulation and ATP Requirement for Caspase-8 and Caspase-3 Activation during CD95- and Anticancer Drug-induced Apoptosis

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Summary

Apoptosis is induced by different stimuli, among them triggering of the death receptor CD95, staurosporine, and chemotherapeutic drugs. In all cases, apoptosis is mediated by caspases, although it is unclear how these diverse apoptotic stimuli cause protease activation. Two regulatory pathways have been recently identified, but it remains unknown whether they are functionally independent or linked to each other. One is mediated by recruitment of the proximal regulator caspase-8 to the death receptor complex. The other pathway is controlled by the release of cytochrome c from mitochondria and the subsequent ATP-dependent activation of the death regulator apoptotic protease-activating factor 1 (Apaf-1). Here, we report that both pathways can be dissected by depletion of intracellular ATP. Prevention of ATP production completely inhibited caspase activation and apoptosis in response to chemotherapeutic drugs and staurosporine. Interestingly, caspase-8, whose function appeared to be restricted to death receptors, was also activated by these drugs under normal conditions, but not after ATP depletion. In contrast, inhibition of ATP production did not affect caspase activation after triggering of CD95. These results suggest that chemotherapeutic drug-induced caspase activation is entirely controlled by a receptor-independent mitochondrial pathway, whereas CD95-induced apoptosis can be regulated by a separate pathway not requiring Apaf-1 function.

Key words: anticancer drugs • apoptosis • ATP • CD95 (APO-1/Fas) • cytochrome c

Apoptosis is a highly conserved process which can be triggered by a broad range of physiological and pathological conditions. Recent evidence suggests that most proapoptotic stimuli induce the activation of a family of intracellular cysteine proteases called caspases (1, 2). These proteases are synthesized as inactive proenzymes which, upon proteolytic cleavage at aspartate residues, form an active complex composed of two heterodimeric subunits. Caspases lead to the proteolysis of a number of cellular substrates, a process which finally results in the apoptotic collapse of the cell.

One of the best-defined apoptotic pathways is mediated by the death receptor CD95 (APO-1/Fas; references 3–5). Triggering of CD95 by its natural ligand CD95L or agonistic antibodies induces the formation of a death-inducing signaling complex (DISC) consisting of the adaptor protein Fas-associated death domain protein (FADD [MORT-1])

and procaspase-8 (FADD-like IL-1 β -converting enzyme [FLICE, Mch5]) (6–9). DISC formation is mediated by two homophilic protein interaction domains: FADD contains a COOH-terminal death domain (DD), which couples to the DD of the intracellular part of CD95. FADD, in addition, contains an NH₂-terminal so-called death effector domain (DED), which binds to one of the DEDs of caspase-8. Further downstream, caspase-8 triggers the proteolytic activation of other caspases and cleavage of cellular substrates.

Furthermore, recent studies have shown that mitochondria play an important role in the regulation of apoptosis. An early event in this process is the translocation of cytochrome c from mitochondria into the cytosol, which is inhibited by antiapoptotic proteins of the Bcl-2 family (10, 11). In the cytosol, cytochrome c interacts with apoptotic protease-activating factor 1 (Apaf-1), the mammalian homologue of the *Caenorhabditis elegans* cell death regulator Ced-4 (12–14). A second cofactor required for Apaf-1 function is dATP (15). Binding of these two components presumably leads to a conformational change in Apaf-1 and

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exposes the so-called caspase recruitment domain (CARD). This region serves as protein interface by binding to caspases that have a similar domain at their NH₂ terminus (16). A CARD motif has been identified in caspase-1, -2, and -9, and caspase-8 contains a long prodomain that may exert a similar regulatory function.

A redistribution of cytochrome c into the cytosol is observed in a variety of apoptotic conditions, such as CD95 ligation, or treatment of cells with staurosporine and chemotherapeutic drugs (10, 11, 17–19). However, it is currently unclear whether the mitochondria-controlled pathway functions independently or is interconnected and required for the CD95 pathway. It has been recently proposed that anticancer drug-induced apoptosis occurs through the CD95 pathway (20, 21). A variety of drugs have been observed to induce upregulation of CD95L expression, followed by the subsequent induction of CD95-dependent apoptosis. However, there are also reports indicating that antitumor drugs induce apoptosis in the absence of CD95 engagement (22–24).

In this study, we dissected the regulation of caspase activation in response to CD95 ligation and treatment of cells with chemotherapeutic drugs. We demonstrate that, similar to CD95, chemotherapeutic drugs are able to induce activation of the initiator caspase-8 and the effector caspase-3, yet drug-induced caspase activation did not require the CD95 receptor/ligand system. We also investigated the contribution of the mitochondria/Apaf-1 pathway to apoptosis induced by CD95, anticancer drugs, and staurosporine. For this purpose, cells were depleted of ATP, which is required for Apaf-1 function and mitochondria-controlled apoptosis. We show that inhibition of ATP production completely abolished caspase activation after treatment of cells with staurosporine and anticancer drugs. In contrast, regardless of ATP depletion, CD95-induced caspase activation was not markedly affected. Our data suggest that drug-induced caspase activation is independent of CD95 and involves only the Apaf-1-regulated pathway, whereas CD95-mediated apoptosis does not essentially require mitochondria-controlled processes.

Materials and Methods

Cells and Reagents. The human Jurkat T cell line was maintained in RPMI 1640 medium supplemented with 10% FCS, 10 mM Hepes, and antibiotics (all from GIBCO BRL, Eggenstein, Germany). The CD95-resistant Jurkat subline Jurkat-R was generated by continuous culture in the presence of anti-CD95 mAb (IgG₃, 1 µg/ml; Cell Diagnostica, Münster, Germany) for 6 mo. Etoposide and mitomycin C were obtained from the clinical pharmacy (Medical Clinics, Tübingen, Germany). Doxorubicin, cycloheximide, and oligomycin were purchased from Sigma (Deisenhofen, Germany) and staurosporine from Boehringer Mannheim GmbH (Mannheim, Germany). Mitomycin C was dissolved in methanol, and doxorubicin, etoposide, and staurosporine in ethanol and kept as stock solutions at -70°C. Intracellular ATP was depleted by incubating cells in glucose-free RPMI 1640 medium supplemented with 2 mM pyruvate, 0.1% FCS, and 2.5 µM oligomycin, an inhibitor of F₀F₁-ATPases, in order to pre-

vent production of ATP from both glycolysis and oxidative phosphorylation (25, 26).

Cell Extracts and Immunoblotting. Cleavage of caspases and the caspase substrate poly(ADP-ribose)polymerase (PARP) was detected by immunoblotting. 2 × 10⁶ cells were seeded in 24-well plates and treated with the apoptotic stimuli. After the indicated time periods, cells were washed in cold PBS and lysed in 1% NP-40, 20 mM Hepes, pH 7.9, 350 mM NaCl, 20% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, containing 3 µg/ml aprotinin, 3 µg/ml leupeptin, and 2 mM PMSF. Subsequently, proteins were separated under reducing conditions on an SDS-polyacrylamide gel and electroblotted to a polyvinylidene difluoride membrane (Amersham Buchler GmbH, Braunschweig, Germany). Membranes were blocked for 1 h with 5% nonfat dry milk powder in TBS and then immunoblotted for 1 h with rabbit anti-PARP antibody (1:2,000; Boehringer Mannheim GmbH) and mouse mAbs directed against caspase-8 (1:10 dilution of a hybridoma supernatant; Biomedica, Baesweiler, Germany) and caspase-3 (R&D Systems, Wiesbaden, Germany). Membranes were washed four times with TBS/0.05% Tween 20 and incubated with the respective peroxidase-conjugated secondary antibody for 1 h. After extensive washing, the reaction was developed by enhanced chemiluminescent staining using ECL reagents (Amersham Buchler GmbH).

Cytochrome c Release and In Vitro Caspase Activation. For analysis of cytochrome c release, cells were collected by centrifugation, washed with ice-cold PBS, and resuspended in 5 vol of buffer A containing 250 mM sucrose, 20 mM Hepes, pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin. The cells were homogenized with 15 strokes in a douncer, and the homogenates were centrifuged at 1,000 g to remove cell nuclei. The supernatants were transferred to a fresh tube and centrifuged at 10,000 g for 10 min to deplete mitochondria. The resulting supernatants, designated as cytosolic S10 fraction, from each sample were loaded on a 12%-SDS polyacrylamide gel. Cytochrome c release was analyzed by immunoblotting with the mouse mAb 7H8.2C12 (PharMingen Europe, Hamburg, Germany). To evaluate the ability of cytochrome c to activate caspases in cytosolic extracts, 150 µg of an S10 fraction was incubated with 1.25 µM bovine cytochrome c (Sigma) and 1 mM dATP at 30°C in a final volume of 25 µl for the indicated time periods. At the end of the incubation, the reaction mixture was loaded on an SDS-polyacrylamide gel and analyzed for caspase-3 and -8 cleavage as described above.

Measurement of Apoptosis. For determination of apoptosis, 3 × 10⁴ cells per well were seeded in microtiter plates and treated for the indicated time points with anti-CD95 or the chemotherapeutic agents. Leakage of fragmented DNA from apoptotic nuclei was measured as described previously (27). In brief, apoptotic nuclei were prepared by lysing cells in a hypotonic buffer (1% sodium citrate, 0.1% Triton X-100, 50 µg/ml propidium iodide) and subsequently analyzed by flow cytometry. Nuclei to the left of the 2N peak containing hypodiploid DNA were considered as apoptotic. In addition, cell death was determined by propidium uptake into cells. All flow cytometry analyses were performed on a FACScalibur® (Becton Dickinson GmbH, Heidelberg, Germany) using CellQuest analysis software.

Measurement of Intracellular ATP. The cellular ATP content was determined using a bioluminescence assay (Sigma). 10⁶ cells were treated with the proapoptotic stimuli in the presence and absence of oligomycin. After the indicated times, cells were washed with PBS, lysed in 0.5% Triton X-100, 10 mM Tris-

HCl, pH 7.5, 1 mM EDTA and incubated for 10 min on ice. After removal of cell debris, ATP content was measured with a luciferin/luciferase assay using a luminometer (model ML2200; Dynatech Deutschland GmbH, Denkendorf, Germany). The ATP content was calculated using an internal standard, and the data were calculated as the mean from three experiments.

Results and Discussion

Caspase-8 has been identified as the most proximal caspase which is recruited to the DISC by its unique DED (7–9). In most cells, caspase-8 is synthesized as two isoforms of ~55 kD (caspase-8/a and -8/b) which, after formation of intermediate cleavage products of 43 and 41 kD, are processed to a p18 and p10 heterodimer (28, 29). As assessed with an anti-p18 antibody, treatment of Jurkat T lymphocytes with agonistic anti-CD95 antibody resulted in the cleavage of procaspase-8 into its characteristic intermediate fragments and the active p18 subunit (Fig. 1). Interestingly, an identical cleavage pattern was obtained after treatment with other apoptotic stimuli, including the protein kinase inhibitor staurosporine, the anticancer drugs doxorubicin and mitomycin C, and cycloheximide, an inhibitor of the protein synthesis (Fig. 1). Because apoptosis mediated by anticancer drugs has been proposed to induce the expression of CD95L and elicit cell death by subsequent CD95 interaction (20, 21), we also used the subclone Jurkat-R, which was selected for resistance to CD95 signaling. Incubation with staurosporine, anticancer drugs, and cycloheximide induced caspase-8 cleavage to a similar extent in CD95-resistant and -sensitive cells, whereas virtually no cleavage was observed by anti-CD95 in the CD95-resistant Jurkat-R cell line (Fig. 1). Additional experiments revealed that anticancer drugs induced caspase-8 activation with similar kinetics and dose dependency in both cell types (data not shown). Therefore, these results suggest that caspase-8 activation is not restricted to apoptosis mediated by death receptors, but is also induced by other proapoptotic stimuli in a CD95-independent pathway.

It has been suggested that the cellular energy charge acts as control point of cell death by either necrosis or apoptosis, and that ATP is necessary for the execution of the apop-

totic program (25, 26, 30). In conjunction with cytochrome c, ATP is required for the function of Apaf-1, which initiates caspase activation in the mitochondria-controlled pathway (12, 13, 15). Therefore, we investigated whether manipulation of intracellular ATP levels can be used to analyze the involvement of the cytochrome c/Apaf-1 pathway during apoptosis triggered by certain stimuli. ATP levels were depleted in Jurkat cells by incubating cells in glucose-free medium and oligomycin, an inhibitor of mitochondrial F_0F_1 -ATPases. Under these conditions, ATP levels declined rapidly within 60 min. Treatment of Jurkat cells with anti-CD95 induced a marked cleavage of caspase-8 within 1 h that was not affected upon depletion of ATP by oligomycin (Fig. 2 A). Furthermore, activation of caspase-3 was observed with slightly delayed kinetics in both ATP-depleted and nondepleted cells. We also analyzed the cleavage of PARP, an enzyme involved in DNA repair, which has been shown to serve as a substrate for caspase-3 (31). Fig. 2 demonstrates that PARP, a 116-kD protein, was cleaved into its characteristic 89-kD fragment with similar kinetics as caspase-3, regardless of the presence or absence of ATP.

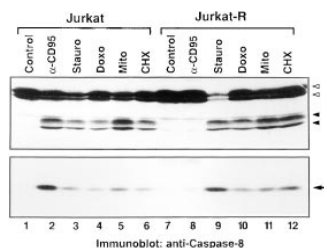


Figure 1. Various apoptotic stimuli induce caspase-8 activation independently of the CD95 pathway. 10^6 CD95-sensitive Jurkat or CD95-resistant Jurkat-R cells were cultured in normal growth medium and then either left untreated (*Control*) or treated with anti-CD95 (1 μ g/ml, 3 h), staurosporine (*Stauro*; 2.5 μ M, 6 h), doxorubicin (*Doxo*; 2 μ g/ml,

12 h), mitomycin C (*Mito*; 25 μ g/ml, 8 h), or cycloheximide (*CHX*; 25 μ g/ml, 6 h). Cellular proteins were separated by SDS-PAGE, and proteolytic processing of caspase-8 was detected by immunoblotting with an antibody against the caspase-8 p18 subunit. *Open arrowheads*. The two isoforms of procaspase-8/a and -8/b, which are cleaved into the intermediate forms p43 and p41 (*filled arrowheads*) and finally processed to the active p18 subunit (*arrow*).

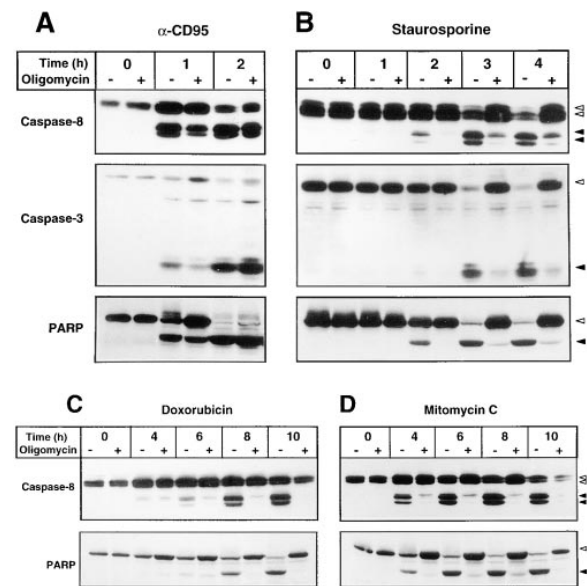


Figure 2. ATP depletion differentially affects caspase activation and cleavage of the caspase substrate PARP in response to anti-CD95 and drug treatment. Jurkat cells were kept in glucose-free medium in either the absence or presence of 2.5 μ M oligomycin, and triggered with different apoptotic stimuli. Anti-CD95 (A), staurosporine (B), doxorubicin (C), and mitomycin C (D) were used at the drug concentrations described in the legend to Fig. 1. Total cell lysates were subjected to 15% SDS-PAGE to detect caspase-8 processing, and to 8–15% gradient SDS-PAGE to detect cleavage of caspase-3 and PARP. Separated proteins were immunoblotted with caspase-8, caspase-3, and PARP-specific antibodies. *Top*, Caspase-8 cleavage; only sections of the immunoblots indicating the cleaved intermediate forms (p43 and p41) of caspase-8/a and -8/b are shown. *Middle*, Processing of caspase-3; the immunoblots denote the position of procaspase-3 and its p17 active subunit. *Bottom*, PARP cleavage; *filled arrowheads* indicate the uncleaved p116 and *open arrowheads* the cleaved p89 form of PARP.

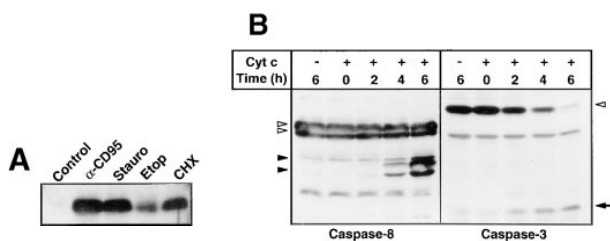


Figure 3. Release of cytochrome c upon induction of apoptosis and in vitro caspase activation. (A) Release of cytochrome c into the cytosol. Jurkat cells were either left untreated in normal culture medium or incubated with anti-CD95 (1 $\mu\text{g}/\text{ml}$, 3 h), staurosporine (*Stauro*; 2.5 μM , 6 h), etoposide (*Etop*; 25 $\mu\text{g}/\text{ml}$, 6 h), or cycloheximide (*CHX*; 25 $\mu\text{g}/\text{ml}$, 5 h). Cells were then homogenized, and the S10 fraction depleted of mitochondria was subjected to 12% SDS-PAGE. The release of cytochrome c into the cytosol was determined by immunoblotting. (B) In vitro activation of caspases by cytochrome c. Aliquots of an S10 fraction (150 μg) were incubated for the indicated time with 1.25 μM cytochrome c in the presence of dATP. A control fraction was incubated for 6 h in the absence of cytochrome c. Proteolytic processing of caspase-8 (left) and caspase-3 (right) was determined by immunoblotting.

In contrast to the CD95 pathway, ATP depletion strongly inhibited caspase-8 and -3 activation as well as PARP cleavage in response to the receptor-independent apoptotic stimuli. After treatment with staurosporine, caspase-8 activation was detectable within 2 h and maximal after 3 h. Inhibition of ATP production strongly prevented caspase-8 activation (Fig. 2 B). Similar inhibitory effects were obtained when caspase-3 and PARP cleavage were analyzed. In addition to staurosporine, caspase activation in response to chemotherapeutic drugs was almost completely abolished in ATP-depleted cells. Doxorubicin-induced caspase-8 activation started within 4–6 h, and was most pronounced after 10 h (Fig. 2 C). Interestingly, similar to CD95, caspase-3 activation and PARP cleavage followed caspase-8 processing. However, in contrast to CD95, activation of both caspases was strongly abrogated by oligomycin. Almost identical results were obtained during apoptosis mediated by mitomycin C (Fig. 2 D) or etoposide (data not shown). Therefore, the differential requirement of ATP for caspase activation by either CD95 or chemotherapeutic drugs indicates distinct regulatory pathways for the two types of apoptosis.

Recent evidence has demonstrated that mitochondria participate in the execution of apoptosis by release of cytochrome c (10, 11, 17–19). Binding of cytochrome c to Apaf-1 results in the cleavage of procaspase-9 or other caspases, which in turn activate caspase-3 (13). Preparation of cytosolic fractions confirmed that anti-CD95, staurosporine, etoposide, and cycloheximide (Fig. 3 A), as well as the other chemotherapeutic drugs (data not shown), induced the redistribution of cytochrome c. It has been shown that caspase-9 and -3 can be activated in cell-free systems upon incubation of cytosolic fractions with cytochrome c and dATP (12, 13, 15). Because caspase-8 activation so far has been observed only after formation of an FADD-containing death receptor complex, we investigated whether caspase-8 can be also activated in vitro by the addition of

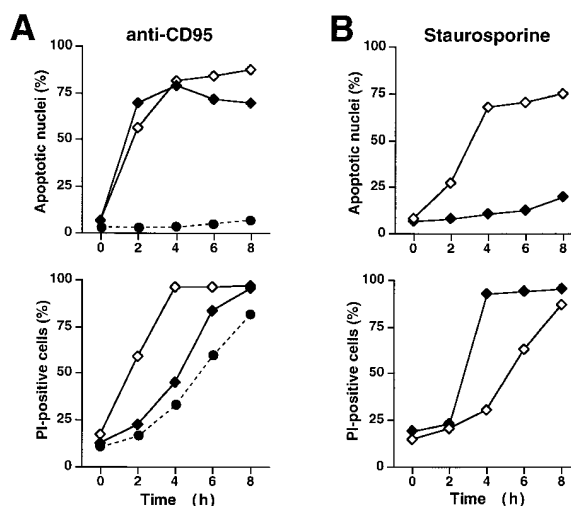


Figure 4. Effects of ATP depletion on CD95- and staurosporine-induced apoptosis. Jurkat cells were either left untreated (*open symbols*) or pretreated for 45 min with oligomycin (*filled symbols*) and then incubated with anti-CD95 (1 $\mu\text{g}/\text{ml}$), staurosporine (2.5 μM), or medium control (*dashed line*). (A) Formation of apoptotic nuclei was determined by flow cytometry of hypodiploid DNA. (B) Membrane damage was determined by the uptake of propidium iodide into cells and flow cytometry. (C) Measurement of intracellular ATP. Extracts of control cells and cells treated for 2 h with anti-CD95 or staurosporine (*Stauro*) in the presence and absence of oligomycin were analyzed for ATP content using a bioluminescence assay. Data represent the mean of three experiments.

cytochrome c. Indeed, incubation of cytosolic extracts with cytochrome c resulted in a potent processing of caspase-8 that was even more pronounced than caspase-3 cleavage by cytochrome c (Fig. 3 B). Therefore, the data demonstrate that the initiator caspase-8 can be activated not only at the DISC level but also by cytochrome c. Future studies will show whether caspase-8 activation is mediated directly by ATP and cytochrome c-bound Apaf-1 or by another proximal caspase, such as caspase-9.

The above experiments suggested that drug-induced caspase-8 activation is CD95-independent and entirely controlled by an ATP-dependent step, most probably Apaf-1 activation. Therefore, we investigated the effects of ATP depletion on apoptotic DNA fragmentation and cell death. Stimulation of Jurkat cells with anti-CD95 resulted in the rapid formation of hypodiploid DNA, which was comparable in ATP-depleted cells and cells kept under normal growth conditions (Fig. 4 A). In contrast, staurosporine-induced nuclear apoptosis was completely abrogated when ATP generation was inhibited (Fig. 4 B), consistent with its effect on caspase activation. However, ATP depletion did not prevent final cell death, as assessed by membrane uptake of propidium iodide into cells. Instead, in the presence of oligomycin, cell death occurred by necrosis. Very similar effects of ATP depletion were found

when nuclear apoptosis and cell death in response to chemotherapeutic drugs were analyzed (data not shown). Fig. 4 C demonstrates that treatment of cells with oligomycin resulted in a strong decrease in ATP production. However, anti-CD95 and staurosporine themselves only slightly affected intracellular ATP levels in early stages of apoptosis.

In summary, manipulation of intracellular ATP levels is a useful means by which to discriminate between the involvement of distinct apoptotic pathways. Although ATP may affect other events, such as chromatin condensation (32), the requirement for ATP can be most likely attributed to its stimulatory function on Apaf-1 in the mitochondrial

pathway. Our data reveal that caspase activation induced by staurosporine and chemotherapeutic drugs is entirely mediated by this signaling pathway and does not require CD95 interaction as previously proposed (20, 21). In contrast, CD95 signaling, although it activates mitochondria (33, 34), can circumvent this evolutionary conserved pathway in order to activate caspases. Whether this bypass holds true for all cell types or whether it is dependent on the degree of DISC formation (34) remains to be shown. Our findings convincingly demonstrate that the cytochrome c/Apaf-1 pathway and receptor-triggered signaling events are not necessarily linked to each other.

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