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 receptor 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-1β-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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exerts the so-called caspase recruitment domain (CARD). This region serves as protein interface by binding to caspases that have a similar domain at their N 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 (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 CD95, chemotherapeutic drugs are able to induce activation in response to CD95 ligation and 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 agents. Leakage of fragmented DNA from apoptotic nuclei was determined using a bioluminescence assay (Sigma). 10,000 cells were loaded on a 12%-SDS polyacrylamide gel and analyzed for caspase-3 and -8 cleavage as described above.

Measurement of Apoptosis. For determination of apoptosis, 3 × 10⁶ cells per well were seeded in microtiter plates and treated for the indicated time periods 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.

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-
forms of (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-CD 95 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 apoptotic 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$_{0}$/F$_{1}$-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. 2A). 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](image1.png)

**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 (C control) or treated with anti-CD95 (1 $\mu$g/ml, 3 h), staurosporine (Stauro; 2.5 $\mu$M, 6 h), doxorubicin (Doxo; 2 $\mu$g/ml, 12 h), mitomycin C (Mito; 25 $\mu$g/ml, 8 h), or cycloheximide (CHX; 25 $\mu$g/ml, 6 h). Cellular proteins were separated by SDS-PAGE, and zymolytic 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](image2.png)

**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 $\mu$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% SDSPAGE to detect caspase-8 processing, and to 8–15% gradient SDS-PAGE to detect cleavage of caspase-3 and PARP. Separated proteins were immunobotted 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.
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. 2B). 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. 2C). 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. 2D) 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. 3A), 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 caspase 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 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. 3B). 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. 4A). In contrast, staurosporine-induced nuclear apoptosis was completely abrogated when ATP generation was inhibited (Fig. 4B), 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.

References

1. Cohen, G.M. 1997. Caspases: the executors of apoptosis. Biochem. J. 326:1–16.
2. Nicholson, D.N., and N.A. Thornberry. 1997. Caspases: killer proteases. Trends Biochem. Sci. 22:299–306.
3. Schulze-Osthoff, K., D. Ferrari, M. Los, S. Wesselborg, and M.E. Peter. 1998. Apoptosis signaling by death receptors. Eur. J. Biochem. 254:439–459.
4. Krammer, P.H., J. Dhein, H. Walczak, I. Behrmann, S. Mariani, B. Mati, M. Fath, P.T. Daniel, E. Knipping, M.O. Westendorp, et al. 1994. The role of APO-1-mediated apoptosis in the immune system. Immunol. Rev. 142:175–191.
5. Nagata, S. 1997. Apoptosis by death factor. Cell. 88:335–365.
6. Kischkel, F.C., S. Hellbardt, I. Behrmann, M. Germer, M. Pawlita, P.H. Kramer, and M.E. Peter. 1995. Cytoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J. 14:5579–5588.
7. Mizuo, M., A.M. Chinnaian, F.C. Kischkel, K. O’Rourke, A. Shevchenko, J.N. Ci, P. Scaffidi, J.B. Bretz, M. Zhang, R. Gentz, et al. 1996. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. Cell. 85:817–827.
8. Boldin, M.P., T.M. Goncharov, Y.V. Goltsev, and D. Wallach. 1996. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell. 85:803–815.
9. Srinivasula, S.M., M. Ahmad, T. Fernandez-Alnemri, G. Litwack, and E.S. Alnemri. 1996. Molecular ordering of the Fas apoptotic pathway: the Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases. Proc. Natl. Acad. Sci. U.S.A. 93:14486–14491.
10. Kluck, R.M., E. Bossy-Wetzel, D.R. Green, and D.D. Newmeyer. 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science. 275:1132–1136.
11. Yang, J., X. Liu, K. Bhalla, C.N. Kim, A.M. Ibrado, J. Cai, T.J. Peng, D.P. Jones, and X. Wang. 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science. 275:1129–1132.
12. Zou, H., W.J. Henzel, X. Liu, A. Lutschg, and X. Wang. 1997. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell. 90:405–413.
13. Li, P., D. Nijhawan, I. Budihardjo, S.M. Srinivasula, M. Ahmad, E.S. Alnemri, and X. Wang. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade. Cell. 91:479–489.
14. Pan, G., K. O’Rourke, and V.M. Dixit. 1998. Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. J. Biol. Chem. 273:5841–5845.
15. Liu, X., C.N. Kim, J. Yang, R. Jemmerson, and X. Wang. 1996. Induction of apoptotic program in cell-free extracts requirement for dATP and cytochrome c. Cell. 86:147–157.
16. Hofmann, K., P. Bucher, and J. Tschopp. 1997. The CARD domain: a new apoptotic signalling motif. Trends Biochem. Sci. 22:155–156.
17. Krippner, A., M. Matsuno-Yagi, R.A. Gottlieb, and B.M. Babior. 1996. Loss of function of cytochrome c in Jurkat cells undergoing Fas-mediated apoptosis. J. Biol. Chem. 271:21629–21636.
18. Adachi, S., A.R. Cross, B.M. Babior, and R.A. Gottlieb. 1997. Bcl-2 and the mitochondrial membrane in the inactivation of cytochrome c during Fas-mediated apoptosis.

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19. Kharbanda, S., P. Pandey, L. Schofield, S. Israels, R. Ronciske, K. Yoshida, A. Bhatti, Z.-M. Yuan, S. Saxena, R. Weichselbaum, et al. 1997. Role of Bcl-x as an inhibitor of cytosolic cytochrome c accumulation in DNA damage-induced apoptosis. Proc Natl Acad Sci USA. 94:6939–6942.

20. Friessen, C., I. Herr, P.H. Krammer, and K.M. Debatin. 1996. Involvement of the CD95 (APO-1/Fas) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nat Med. 2:574–577.

21. Müller, M., S. Strand, H. Hug, E.M. Heinemann, H. Walczak, W.J. Hofmann, W. Stremmel, P.H. Krammer, and P.R. Galle. 1997. Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. J Clin Invest. 99:403–413.

22. Eischen, C.M., T.J. Kottke, L.M. Martins, G.S. Basu, J.S. Tung, W.C. Earnshaw, P.J. Leibson, and S.H. Kaufman. 1997. Comparison of apoptosis in wild-type and Fas-resistant cells: chemotherapy-induced apoptosis is not dependent on Fas/Fas ligand interactions. Blood. 90:935–943.

23. Gamen, S., A. Anel, P. Laserra, M.A. Alava, M.J. Martinez-Lorenzo, A. Pineiro, and J. Navalg. 1997. Doxorubicin-induced apoptosis in human T-cell leukemia is mediated by caspase-3 activation in a Fas-independent way. FEBS Lett. 417:360–364.

24. Yeh, W.C., J.L. Pompa, M.E. Mccurrach, H.B. Shu, A.J. Elia, A. Shahnian, M. Ng, A. Wakeham, W. Khoo, K. Mitchell, et al. 1998. FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. Science. 279:1954–1958.

25. Leist, M., B. Single, A.F. Castoldi, S. Kuhnle, and P. Nicotera. 1997. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med. 185:1481–1486.

26. Eguchi, Y., S. Shimizu, and Y. Tajiimoto. 1997. Intracellular ATP levels determine cell death fate by apoptosis or necrosis. Cancer Res. 57:1835–1840.

27. Nicoletti, I., G. Migliorati, M.C. Pagliacci, F. Grignani, and C. Riccardi. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods. 139:271–279.

28. Medema, J.P., C. Scaffidi, F.C. Kischkel, A. Shevchenko, M. Mann, P.H. Krammer, and M.E. Peter. 1997. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). EMBO J. 16:2794–2804.

29. Scaffidi, C., J.P. Medema, P.H. Krammer, and M.E. Peter. 1997. FLICE is predominantly expressed as two functionally active isoforms, caspase-8/a and caspase-8/b. J Biol Chem. 272:26953–26958.

30. Richter, C., M. Schweizer, A. Cossarizza, and C. Franceschi. 1996. Control of apoptosis by ATP level. FEBS Lett. 378:107–110.

31. Tewari, M., L.T. Quan, K. O'Rourke, S. Desnoyers, Z. Zeng, D.R. Beidler, G.G. Poirier, G.S. Salvesen, and V.M. Dixit. 1995. Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell. 81:801–809.

32. Kass, G.E.N., J.E. Eriksson, M. Weis, S. Orrenius, and S.C. Chow. 1996. Chromatin condensation during apoptosis requires ATP. Biochim Biophys Acta. 1318:749–752.

33. Kroemer, G. 1997. Mitochondrial control of apoptosis. Immunol Today. 18:44–51.

34. Scaffidi, C., S. Fulda, A. Srinivasan, C. Friessen, F. Li, K.J. Tomasselli, K.M. Debatin, P.H. Krammer, and M.E. Peter. 1998. Two CD95 (APO-1/Fas) signaling pathways. EMBO J. 17:1675–1687.