Distinct Caspase Cascades Are Initiated in Receptor-mediated and Chemical-induced Apoptosis*

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Release of cytochrome c is important in many forms of apoptosis. Recent studies of CD95 (Fas/APO-1)-induced apoptosis have implicated caspase-8 cleavage of Bid, a BH3 domain-containing proapoptotic member of the Bcl-2 family, in this release. We now demonstrate that both receptor-induced (CD95 and tumor necrosis factor) and chemical-induced apoptosis result in a similar time-dependent activation of caspases-3, -7, -8, and -9 in Jurkat T cells and human leukemic U937 cells. In receptor-mediated apoptosis, the caspase inhibitor, benzyloxy carbonyl-Val-Ala-Asp fluoromethyl ketone (Z-VAD.FMK), inhibits apoptosis prior to commitment to cell death by inhibiting the upstream activator caspase-8, cleavage of Bid, release of mitochondrial cytochrome c, processing of effector caspases, loss of mitochondrial membrane potential, and externalization of phosphatidylserine. However, Z-VAD.FMK inhibits chemical-induced apoptosis at a stage after commitment to cell death by inhibiting the initiator caspase-9 and the resultant postmitochondrial activation of effector caspases. Cleavage of Bid but not release of cytochrome c is blocked by Z-VAD.FMK demonstrating that in chemical-induced apoptosis cytochrome c release is caspase-independent and is not mediated by activation of Bid. We propose that caspases form an integral part of the cell death-inducing mechanism in receptor-mediated apoptosis, whereas in chemical-induced apoptosis they act solely as executioners of apoptosis.

Apoptosis is a major form of cell death characterized by a series of stereotypic morphological features. It occurs in two phases, an initial commitment phase followed by an execution phase involving the condensation and fragmentation of nuclear chromatin, dilation of the endoplasmic reticulum, and alterations to the cell membrane resulting in recognition and subsequent phagocytosis of the cell (1, 2). Caspases, a family of cysteine proteases, play a critical role in the execution phase of apoptosis and are responsible for many of the biochemical and morphological changes associated with apoptosis (3, 4). It has been proposed that “initiator” caspases with long prodomains, such as caspase-8 (MACH/FLICE/Mch5), either directly or indirectly activate “effector” caspases, such as caspases-3, -6, and -7 (3, 5, 6). These effector caspases then cleave intracellular substrates, such as poly(ADP-ribose) polymerase (PARP)1 and lamins, during the execution phase of apoptosis. Caspase-8 is the most apical caspase in CD95 (Fas/APO-1)-induced apoptosis (7, 8). Triggering of the CD95 receptor with its cognate ligand or agonistic antibody results in receptor trimerization and recruitment of CD95 receptor-associated protein with death domains (FADD/MORT1), which in turn binds to the death effector domains in the N-terminal region of caspase-8, resulting in its activation. As caspase-8 can activate all known caspases in vitro (6), it is a prime candidate for an initiator caspase in many forms of apoptosis in addition to CD95-induced apoptosis. Procaspase-9 has also been proposed as an initiator caspase; in the presence of dATP and cytochrome c, its long N-terminal domain interacts with Apaf-1 resulting in the activation of caspase-9 (9, 10). Active caspase-9 can then activate the effector caspases-3, -6, and -7 (10, 11). Thus there are at least two major mechanisms by which a caspase cascade resulting in the activation of effector caspases may be initiated as follows: one involving caspase-8 and the other involving caspase-9 as the most apical caspase.

Cytochrome c, which is usually present in the mitochondrial intermembrane space, is released into the cytosol following the induction of apoptosis by many different stimuli including CD95, tumor necrosis factor (TNF), and chemotherapeutic and DNA-damaging agents (12–14). Mitochondria have been proposed to act as an amplifier in CD95-induced apoptosis when activation of caspase-8 cleaves a cytosolic substrate leading to release of cytochrome c (15, 16). Release of mitochondrial cytochrome c and activation of caspase-3 is blocked by anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-XL, and promoted by proapoptotic members, such as Bax and Bak (13, 17, 18). In chemical- or irradiation-induced apoptosis, cytochrome c release appears to be caspase-independent as it is not inhibited by the broad spectrum cell-permeable caspase inhibitor, Z-VAD.FMK (13, 17, 19, 20). Mechanisms for the release of mitochondrial cytochrome c include opening of a mitochondrial permeability transition pore, the presence of a specific channel for cytochrome c in the outer membrane, or mitochondrial swelling and rupture of the outer membrane but without loss of mitochondrial membrane potential (14). None of these mechanisms appears generally applicable, as release of cytochrome c occurs in cells with normal mitochondrial membrane potential (13, 17) and by a mechanism independent of rupture of the

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1 The abbreviations used are: PARP, poly(ADP-ribose) polymerase; Z-VAD.FMK, benzyloxy carbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone; Z-IETD.CHO, benzyloxy carbonyl-Ile-Glu-Thr-Asp aldehyde; Z-DEVD.AFC, benzyloxy carbonyl-Asp-Glu-Val-Ala amino fluoromethyl coumarin; TNF, tumor necrosis factor; PS, phosphatidyl serine; DiOC6(3), 3,3'-dihexyloxacarbocyanine iodide; DED, death effector domain.
outer mitochondrial membrane (20). Two recent studies have highlighted another possible mechanism of mitochondrial cytochrome c release, involving Bid, a BH3 domain-containing proapoptotic Bcl-2 family member. Cleavage of Bid by caspase-8 results in translocation of the cleaved Bid to the mitochondria where it induces the release of cytochrome c, being 500-fold more potent than Bax (21, 22). The BH3 domain of Bid is essential both for its proapoptotic activity and its ability to induce the release of cytochrome c (22, 23).

In this study we address the order in which caspases are activated in receptor-mediated and chemical-induced apoptosis. Our data support the hypothesis that caspase-8 and caspase-9 are the apical caspases in receptor-mediated and chemical-induced apoptosis, respectively. In chemical-induced apoptosis, cytochrome c release is caspase-independent and is not mediated by cleavage of Bid in contrast to receptor-mediated apoptosis. We propose that caspases act solely as executioners of apoptosis in chemical-induced apoptosis, whereas in receptor-mediated apoptosis they also form an integral part of the cell death-inducing mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**—Media and serum were purchased from Life Technologies, Inc. (Paisley, UK). Z-VAD.FMK and Z-DEVD.AFC were from Enzyme Systems Inc. (Dublin, CA), and Z-IEDOCHO was kindly provided by Professor L. Rubin (Eisai London Research, London, UK). Anti-CtD95 monoclonal antibody was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Annexin V/FluTC kit was from Bender MedSystems (Vienna, Austria). DiOC6(3) was purchased from Molecular Probes (Eugene, OR). All other chemicals and human recombinant TNF-α were from Sigma (Poole, UK).

**Cell Culture and Quantification of Apoptosis by Flow Cytometry**—Jurkat T cells (clone 6B-1) were obtained from ECACC and cultured in RPMI 1640 containing 10% fetal bovine serum and 1% Glutamax. Jurkat T cells (clone E6-1) were obtained from ECACC and cultured in RPMI 1640 containing 10% fetal bovine serum and 1% Glutamax. All other chemicals and human recombinant TNF-α were from Sigma (Poole, UK).

**Preparation of Cell Lysates for in Vitro Caspase Activation Studies**—Jurkat T cell lysates were prepared as described previously (19). A shorter exposure of the film showed that the proform of caspase-9 was present primarily as its intact 32-kDa proform and two subunits. An antibody to caspase-9 was also raised, which verified that the antibody recognized intact procaspase-9 and the p43 and p18 subunits. An antibody to caspase-9 was also raised, which recognized both the inactive proform and the activated caspase-9 (Figs. 1 and 2). In Jurkat T cells, caspase-3 was present primarily as its intact 32-kDa proform (Fig. 1A, lane 1). Induction of both chemical- and receptor-mediated apoptosis resulted in loss of the proform of caspase-3 and appearance of three immunoreactive fragments of ~20 kDa (p20), ~19 kDa (p19), and ~17 kDa (p17), following initial cleavage at Asp175 and then at Asp17 and Asp28 (27). Processing was first detected after 2–3 h treatment with either stimulus (Figs. 1A and 2A, lanes 2–7).

Caspase-7 was present in control Jurkat T cells primarily as a ~35-kDa proform (Fig. 1B, lane 1). Treatment with both anti-CtD95 antibody and etoposide resulted in a time-dependent processing of caspase-7 accompanied by the formation of two major products. These were a ~32-kDa fragment, which probably represents the loss of the prodomain at DSVD235 A, and a ~19-kDa (p19) fragment, which corresponds to the catalytically active large subunit (Figs. 1B and 2B, lanes 2–7) followed by cleavage at IQAD198 S (27). Processing of caspase-7 was first observed 2 h after treatment with either stimulus.

In untreated Jurkat T cells, caspase-8 was present primarily as two isoforms of ~55 kDa (Fig. 1C, lane 1), possibly corresponding to caspase-8a and ~8b (7, 28). Exposure to both anti-CtD95 antibody and etoposide resulted in a time-dependent
processing of caspase-8 initially to two fragments of ~43 and 41 kDa (p43 and p41, respectively), corresponding to cleavage of both caspase-8a and -8b between the large and small subunits. This was followed by the appearance of a p18 subunit resulting from removal of the death effector domains from the 43- and 41-kDa fragments (Figs. 1C and 2C, lanes 2–7) (6, 28). An increase in the processing of caspase-8 was first observed 2 and 2.5 h after CD95 and etoposide treatment, respectively.

Untreated Jurkat cells contained the 46-kDa proform of caspase-9 (Fig. 1D, lane 1), which on induction of apoptosis was processed in a time-dependent manner to yield fragments of ~37 and 35 kDa (p37 and p35) (Figs. 1D and 2D, lanes 2–7), resulting from cleavage at both Asp-315 and Asp-330 (6). The first detectable processing of caspase-9 was evident at 2 h (Figs. 1D and 2D).

Thus, induction of apoptosis was accompanied by the activation of both the activator caspases -8 and -9 and the effector caspases-3 and-7, which all appeared to occur simultaneously making it extremely difficult to distinguish the order in which they were activated. In order to further address this problem, we used the broad-spectrum caspase inhibitor Z-VAD.FMK, which inhibits apoptosis in many but not all systems (3).

**Z-VAD.FMK Inhibits Different Targets in Etoposide- and CD-95-induced Apoptosis**—Slight inhibition of CD95-induced caspase processing was observed at Z-VAD.FMK (0.1 μM), with marked and almost total inhibition at 1.0 and 10 μM, respectively (Fig. 1, A–D, lanes 8–10). Higher concentrations of Z-VAD.FMK were required to inhibit etoposide-induced processing of these caspases (Fig. 2, A—D, lanes 8–10). Z-VAD.FMK (10 and 25 μM) largely but not completely inhibited the processing of caspase-3, -7, and -9 (Fig. 2, A—D, lanes 8 and 9). As caspase-9 processes caspases-3 and -7 (10, 29), these data support the suggestion that one of the important targets of Z-VAD.FMK in etoposide-induced apoptosis may be either the processing or the activity of caspase-9.

**Fig. 2.** Etoposide-induced time-dependent processing of caspases in Jurkat T cells. Jurkat T cells were incubated for the indicated times with etoposide (50 μM) either alone or in the presence of the indicated concentrations of Z-VAD.FMK. Cells were then analyzed by Western blot analysis for the processing of caspase-3 (A), caspase-7 (B), caspase-8 (C), and caspase-9 (D), as described under “Experimental Procedures” and the legend to Fig. 1.

**Fig. 3.** Z-VAD.FMK inhibits the decrease in both mitochondrial membrane potential and cell size in CD95- but not in etoposide-mediated apoptosis. Jurkat T cells were incubated for 6 h with CD95 (50 ng/ml) either alone or in the presence of Z-VAD.FMK (10 μM). Cells were also incubated with etoposide (50 μM) either alone or in the presence of Z-VAD.FMK (25 μM). Apoptosis was quantified by flow cytometric analysis as described in the legend to Fig. 1. Forward light scatter is an indication of cell size. Cells in R2 represent normal cells with low PS exposure and normal cell size; cells in R3 are cells with low PS exposure but with decreased cell size, and cells in R4 have an increase in PS exposure. Cells in R5 have a high mitochondrial membrane potential (ΔΨm), and cells in R6 have a decreased mitochondrial membrane potential. The numbers represent the percentage of cells in the indicated region.
etoposide (50 μM) completely inhibited the processing of caspase-8, which suggested that caspase-8 was activated downstream of caspases-3 and -7. Most importantly these data strongly suggested that the order of caspase activation and the Z-VAD.FMK target(s) were different in etoposide- and CD95-induced apoptosis in Jurkat cells.

The different cellular effects of Z-VAD.FMK on etoposide- and CD95-induced apoptosis in Jurkat cells provided further support for this hypothesis. Both CD95 and etoposide induced apoptosis, as assessed either by an increase in externalization of PS (Fig. 3, B and D) or by a decrease in mitochondrial membrane potential (Fig. 3, G and I). Z-VAD.FMK (10 μM) completely inhibited CD95-induced apoptosis assessed by both these criteria (Fig. 3, C and H). However, Z-VAD.FMK (25 μM) did not inhibit etoposide-induced loss of mitochondrial membrane potential (Fig. 3J) but did inhibit PS exposure (Fig. 3E). Z-VAD.FMK (25 μM) also did not inhibit etoposide-induced decrease in cell size as measured by forward light scatter (Fig. 3, E and J). Taken together, these data suggest that in CD95-induced apoptosis in Jurkat cells the intracellular target of Z-VAD.FMK is most likely the activator caspase-8 acting upstream of mitochondria, whereas in etoposide-induced apoptosis the target(s) is the activation-processing of caspase-9, which is activated downstream of perturbation of mitochondria. In order to substantiate this hypothesis, we examined the effects of Z-VAD.FMK on the release of mitochondrial cytochrome c. Z-VAD.FMK Inhibits Receptor-mediated but Not Chemical-induced Cytochrome c Release—

In agreement with other studies, CD95 induced a time-dependent increase of cytochrome c in the cytosol, most probably due to an increased release of mitochondrial cytochrome c (Fig. 4A). Z-VAD.FMK (10 μM) inhibited this increase (Fig. 4A), further demonstrating that it inhibited a caspase upstream of the mitochondrial changes. Etoposide also induced a time-dependent increase in cytosolic cytochrome c; however, Z-VAD.FMK (25 μM) did not inhibit this increase (Fig. 4B) further supporting the hypothesis that the target of Z-VAD.FMK in etoposide-induced apoptosis is downstream of mitochondria.

In order to determine if such differences between receptor-mediated and chemically induced apoptosis also occurred in other cells, we examined a human lymphoid tumor cell line, U937, which is sensitive to tumor necrosis factor (TNF-α). Both TNF/cycloheximide and etoposide caused an induction of apoptosis in U937 cells, assessed by PS externalization, which was accompanied by an increase in cytosolic cytochrome c, processing of caspase-3, and cleavage of PARP, a commonly used measure of caspase-3-like enzymic activity (Fig. 5, A–C). Z-VAD.FMK completely inhibited TNF/cycloheximide-induced apoptosis assessed by all these criteria. In contrast, in etoposide-induced apoptosis Z-VAD.FMK completely inhibited PS externalization and the cleavage of PARP but only partially inhibited processing of caspase-3 and did not inhibit the increase in cytosolic cytochrome c. Thus Z-VAD.FMK was more effective at blocking the activity rather than the processing of caspase-3. Taken together, the data from both Jurkat and U937 cells support the hypothesis that Z-VAD.FMK inhibits a target upstream of mitochondria in receptor (CD95 or TNF)-mediated apoptosis, whereas in etoposide-induced apoptosis the Z-VAD.FMK target is downstream of mitochondria.

Activation of Jurkat Lysate Results in Initial Processing of Caspase-9—In order to gain further insight into the order of caspase activation in chemical-mediated apoptosis, the processing of various caspases was studied in Jurkat cell lysates, a well established model for understanding postmitochondrial caspase cascades (12). Activation of lysates, which resulted in an increased caspase-3-like DEVDase activity, was accompanied by a time-dependent processing of caspases (Fig. 6). Processing of the effector caspases-3 and -7 was first observed after 5 min, and these caspases were almost completely processed after 60 min (Fig. 6, lanes 1–7). Caspase-9 was very rapidly and extensively processed to both its p37 and p35 fragments, initial processing being observed by 1 min and virtually complete noted at 30 min (Fig. 6, lanes 1–7). In marked contrast, processing of caspase-8 was first observed at 30 min, when a small amount was processed only to its ~41- and 43-kDa fragments (Fig. 6, lanes 1–7). Co-incubation of lysates with two different caspase inhibitors, Z-VAD.FMK or benzyl-oxycarbonyl-Ile-Glu-Thr-Asp-aldehyde (IETDCHO), resulted in a marked inhibition of the processing of all the caspases with
caspase-9 being somewhat less sensitive to inhibition than the other caspases (Fig. 6, lanes 8 and 9). Thus caspase inhibitors may also block the processing of caspases activated by a caspase cascade downstream of mitochondria.

To elucidate further the role of caspases-8 and -9 in the postmitochondrial caspase cascade, they were immunodepleted from lysates, and the subsequent ability of the lysate to be activated was assessed. In the presence of dATP/cytochrome c, lysates from control Jurkat cells exhibited a marked DEVDase activity (Table I). Immunodepletion of caspase-8 caused only a very slight decrease in DEVDase activity demonstrating that caspase-8 contributed little to this activity (Table I). Western blot analysis demonstrated that immunodepletion of caspase-8 resulted only in loss of this caspase but not of other caspases (data not shown). In contrast, immunodepletion of caspase-9 resulted in complete inhibition of DEVDase activity (Table I) without loss of caspases-3, -7, and -8 (data not shown) demonstrating the key role of caspase-9 in the postmitochondrial processing of caspases. Taken together these results lend strong support to the hypothesis that caspase-9 is the first caspase activated in a caspase cascade following perturbation of mitochondrion and release of cytochrome c.

**Z-VAD.FMK Inhibits Cleavage of Bid in Receptor- and Chemical-mediated Apoptosis—**Cleavage of Bid is important for the release of mitochondrial cytochrome c in CD95-induced apoptosis (21, 22). We wished to investigate whether this mechanism of cytochrome c release is important in other receptor-mediated cell deaths or in chemical-mediated apoptosis. Bid was present as an ~26-kDa protein in control Jurkat cells and was cleaved initially to two major fragments of ~15 and 14 kDa (Fig. 7A), most probably following cleavage at LQTD [G and IEAD [S (21, 22). In CD95-mediated apoptosis, cleavage of Bid was first observed at 30 min (Fig. 7A). Z-VAD.FMK inhibited cleavage of Bid in a concentration-dependent manner (Fig. 7A) commensurate with its ability to inhibit caspase processing and release of cytochrome c (Figs. 1 and 4A). Etoposide also induced a time-dependent cleavage of Bid in Jurkat cells with a small amount of fragmentation first being observed at 2 h (Fig. 7B). Z-VAD.FMK inhibited this cleavage in a concentration-dependent manner (Fig. 7B), but it did not inhibit etoposide-induced release of cytochrome c (Fig. 4B). Thus, in etoposide-induced cell death in Jurkat cells, cleavage of Bid was not required for the release of mitochondrial cytochrome c.

In order to extend these studies, we examined cleavage of Bid in other cell types. In U937 cells, induction of apoptosis with either TNF/cycloheximide or etoposide resulted in cleavage of Bid (Fig. 5D, lanes 2 and 4). Z-VAD.FMK prevented cleavage of Bid induced by both apoptotic stimuli (Fig. 5D, lanes 3 and 5); however, it only inhibited cytochrome c release in TNF/cycloheximide- but not in etoposide-induced apoptosis (Fig. 5A). Recently we have shown that in human monocytes THP.1 cells, induction of apoptosis by either etoposide or N-tosyl-l-phenylalanyl chloromethyl ketone (TPCK), a chymotrypsin-like protease inhibitor, is accompanied by the release of mitochondrial cytochrome c, which is not blocked by Z-VAD.FMK (20). Both these chemical stimuli also induced a time-dependent loss of intact Bid accompanied by the formation of fragments of ~15 and 14 kDa, both of which were prevented by Z-VAD.FMK.

| Lysate treatment          | DEVDase (nmol/mg/min) |
|---------------------------|-----------------------|
| Control                   | 14.4                  |
| Preimmune rabbit serum    | 12.2                  |
| Caspase-8 antibody        | 10.1                  |
| Caspase-9 antibody        | 0.07                  |

**TABLE I**

**Immunodepletion of caspase-9 but not caspase-8 inhibits cytochrome c/dATP-dependent activation**

Jurkat lysate was prepared and immunodepleted with either preimmune serum, caspase-8, or caspase-9 antibody, and the proteolytic activity (DEVDase) of the control lysate and the immunodepleted lysates was then measured as described under “Experimental Procedures.”
(data not shown). Thus in three different cellular systems, cleavage of Bid and loss of mitochondrial cytochrome c accompany chemical induction of apoptosis. As Z-VAD.FMK inhibited cleavage of Bid but not the loss of cytochrome c, cleavage of Bid is not responsible for the release of mitochondrial cytochrome c in chemical-induced apoptosis.

**DISCUSSION**

**Z-VAD.FMK Inhibits Receptor-mediated Apoptosis prior to Involvement of Mitochondria**—CD95-mediated apoptosis involves the initial formation of a death-inducing signaling complex (DISC), which is formed following the recruitment of FADD (Fas-associated protein with a death domain) (30). FADD binds through its death effector domain (DED) to one of the two N-terminal DEDs of caspase-8, resulting in its activation (7, 8). Active caspase-8 may induce apoptosis either directly following direct activation of other caspses or indirectly following cleavage of cytosolic factors, such as Bid, leading to involvement of mitochondria and release of cytochrome c, which together with Apaf-1 results in activation of caspase-9 and the effector caspses (6, 16, 21, 22). Interestingly no major differences in CD95-induced apoptosis were observed in T cells from caspase-9−/− and Apaf-1−/− knock-out compared with wild type mice (31–33). However, CD95-induced apoptosis was markedly reduced in Apaf-1−/− compared with wild type embryonic fibroblasts (34) suggesting that the same apoptotic stimulus may utilize different caspses in different cell types. Although mitochondria may be initially bypassed following direct activation of the effector caspases-3 and -7 by caspase-8, these effector caspses may then subsequently disrupt mitochondrial membrane function (Fig. 8). This will lead to a release of intermembrane proteins, such as cytochrome c, resulting in a further activation of caspases and the establishment of a self-amplification loop (35). Thus receptor-mediated apoptosis may initially either directly involve or bypass the mitochondria dependent on the relative concentrations of caspase-8, the effector caspses and cytosolic factors, such as Bid, in a specific cell type (16) (Fig. 8).

Anti-apoptotic members of the Bcl-2 family block mitochondrial cytochrome c release (13, 17) and also block CD95- and TNF-induced apoptosis in some (16, 29, 36) but not all circumstances (37, 38). Thus in a variety of cell types, apoptotic signaling in response to CD95 or TNF stimulation is regulated at least in part by a Bcl-2 and/or Bcl-xL-inhibitable step. In this regard, CD95 has recently been shown to activate two distinct cell death pathways, one involving FADD, which is Bcl-2-insensitive, and one involving Daxx, which is Bcl-2 sensitive (39). Furthermore, Bcl-2 protects mice from the lethal hepatic apoptosis induced by anti-CD95 antibody (40). Taken together these studies demonstrate a physiological role for the involvement of mitochondria in many instances of receptor-mediated apoptosis. In the present study, apoptosis induced by two death receptors, CD95 and TNF, was inhibited by low concentrations of Z-VAD.FMK at a very early stage prior to processing of caspases-3, -7, -8, and -9, cleavage of Bid, release of mitochondrial cytochrome c, loss of mitochondrial membrane potential, and externalization of PS. These data strongly support the hypothesis that the primary target of Z-VAD.FMK in receptor-mediated apoptosis is inhibition of the activation of procaspase-8 prior to any perturbation of mitochondria resulting in the inhibition of all downstream biochemical effects (Fig. 8).

**Z-VAD.FMK Inhibits Chemical-induced Apoptosis Downstream of Mitochondria**—Following release from mitochondria, cytochrome c binds to Apaf-1 resulting in the activation of caspase-9, which in turn activates the effector caspses responsible for the cleavage of many of the substrates associated with the characteristic biochemical and morphological changes of apoptosis (9). In agreement with data from chemical- and radiation-induced apoptosis in other cells (13, 17, 19, 20), Z-VAD.FMK did not inhibit release of mitochondrial cytochrome c in etoposide-induced apoptosis in both Jurkat T and U937 cells. Neither did it completely inhibit processing of caspases-3, -7, and -9 (Figs. 2, 4, and 5). Taken together with the demonstration of the critical role of caspase-9 in cytochrome c-catalyzed caspase activation in lysates (Fig. 6 and Table I), these data support the hypothesis that the Z-VAD.FMK target in chemical-induced apoptosis is the processing or the activity of caspase-9. Recent studies on caspase-9 knock-out mice demonstrate that caspase-9 is a critical upstream activator of a caspase cascade in vivo and in some situations is essential for the processing of caspase-3 (31, 32).

Cleavage of Bid was observed in chemical- as well as receptor-mediated apoptosis. Bid is most likely cleaved by low concentrations of caspase-8 but can be cleaved by higher concentrations of caspase-3 (21). Thus in chemical-induced apoptosis, inhibition of Bid cleavage by Z-VAD.FMK may be due to inhibition of processing/activity of either caspase-3 or -8. In addition, effector caspses may activate caspase-8 at a later stage of the apoptotic process, resulting in cleavage of Bid and release of mitochondrial cytochrome c, thereby further amplifying the apoptotic program (Fig. 8). Most importantly, in contrast to receptor-mediated apoptosis, Z-VAD.FMK blocked cleavage of Bid but not release of cytochrome c (Figs. 4, 5 and 7) demon-
Caspase Activation and Cytochrome c Release

Fig. 8. Scheme for receptor- and chemical-induced apoptosis. Triggering of death receptors, such as CD95 (Fas/Apo-1) or TNF, results in recruitment of adapter molecules, such as FADD and TRADD. The DED of FADD binds to the DED of caspase-8 leading to the oligomerization and activation of caspase-8. Caspase-8 may directly activate the effector caspases, −3 and −7, responsible for many of the biochemical and morphological changes associated with the apoptotic phenotype. Alternatively, caspase-8 can act indirectly by cleaving cytosolic factors, such as Bid, leading to the release of cytochrome c and activation of caspase-9 with subsequent activation of the effector caspases. Active caspases-3 or -7 may then further activate procaspase-9 or alter mitochondrial membrane permeability setting up a self-amplification loop (- - -) or they may cleave DNA fragmentation factor (DFF) or inhibitor of caspase-activated DNase (ICAD) leading to the internucleosomal cleavage of DNA. In chemical-mediated apoptosis, undefined signals lead to perturbation of mitochondria (Mit) and loss of cytochrome c and then activation of caspases.

Caspases Are an Integral Part of the Cell Death-inducing Mechanism in Receptor-mediated Apoptosis whereas in Chemical-induced Apoptosis They Act Solely as Executors—Previous work has demonstrated an increased clonogenic potential of cells treated with CD95 in the presence of caspase inhibitors, such as Z-VAD.FMK (42, 43). Taken together with the present and other studies (7, 8), it suggests that the activator caspase-8 is an integral component of the cell death-inducing mechanism and that in receptor-mediated apoptosis, activation of caspase-8 represents a commitment point to cell death. At a later stage in the apoptotic program other effector caspases are activated and are responsible for the characteristic biochemical and morphological features of the apoptotic phenotype. We cannot exclude a similar role for other activator caspases, such as caspase-10, which like caspase-8 possesses a long N-terminal prodomain with two death effector domains (27).

In chemical-induced apoptosis, Z-VAD.FMK inhibited the activation of caspases and PS externalization but not the loss of mitochondrial cytochrome c (Figs. 2, 4, and 5) or the loss of clonogenic potential (43). Thus in chemical-induced apoptosis, Z-VAD.FMK does not inhibit the commitment to cell death but rather inhibits all the biochemical changes associated with caspase activation occurring following perturbation of mitochondria and loss of cytochrome c. We propose that Z-VAD.FMK inhibits the processing and/or the activity of caspase-9 and thus blocks the subsequent activation of effector caspases, resulting in the block of a self-amplification loop (Fig. 8). Thus in chemical-induced apoptosis, caspase activation occurs after commitment to cell death and is primarily responsible for those stereotypic biochemical and morphological changes commonly associated with the apoptotic phenotype. These results have important clinical implications for the use of caspase inhibitors as potential therapeutic agents. Cells, which are committed to die but with caspases inhibited, may die more slowly and with a different morphology from cells dying with characteristic apoptotic morphology. This hypothesis is compatible with recent reports describing a caspase-independent cell death, such as Bax- or Bak-induced cell death (44, 45). In summary, we have demonstrated that in receptor-mediated apoptosis, Z-VAD.FMK inhibited apoptosis prior to commitment to cell death by inhibiting the upstream activator caspase-8. This resulted in inhibition of cleavage of Bid, loss of mitochondrial cytochrome c, processing of caspases, loss of mitochondrial membrane potential, and externalization of PS. In chemical-induced apoptosis, Z-VAD.FMK inhibited apoptosis downstream of cell death commitment by inhibiting the initiator caspase-9 and the resultant postmitochondrial activation of effector caspases, cleavage of Bid, and PS externalization but not loss of cytochrome c or mitochondrial membrane potential. Thus in chemical-induced apoptosis, a mechanism other than cleavage of Bid is responsible for the release of cytochrome c.

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