

Necrotic Death Pathway in Fas Receptor Signaling

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Abstract. A caspase 8-deficient subline (JB6) of human Jurkat cells can be killed by the oligomerization of Fas-associated protein with death domain (FADD). This cell death process is not accompanied by caspase activation, but by necrotic morphological changes. Here, we show that the death effector domain of FADD is responsible for the FADD-mediated necrotic pathway. This process was accompanied by a loss of mitochondrial transmembrane potential ($\Delta\Psi_m$), but not by the release of cytochrome c from mitochondria. Pyrrolidine dithiocarbamate, a metal chelator and antioxidant, efficiently inhibited the FADD-induced reduction of $\Delta\Psi_m$ and necrotic cell death. When human Jurkat, or its transformants, expressing mouse Fas were treated

with Fas ligand or anti-mouse Fas antibodies, the cells died, showing characteristics of apoptosis. A broad caspase inhibitor (z-VAD-fmk) blocked the apoptotic morphological changes and the release of cytochrome c. However, the cells still died, and this cell death process was accompanied by a strong reduction in $\Delta\Psi_m$, as well as necrotic morphological changes. The presence of z-VAD-fmk and pyrrolidine dithiocarbamate together blocked cell death, suggesting that both apoptotic and necrotic pathways can be activated through the Fas death receptor.

Key words: apoptosis • caspase • Fas • mitochondrial membrane potential • necrosis

Introduction

Based on morphological changes, cell death in mammals is classified into two types: apoptosis and necrosis (Wyllie et al., 1980; Duval and Wyllie, 1986). Apoptosis is characterized by plasma membrane blebbing, condensation, and fragmentation of cells and nuclei, as well as extensive degradation of chromosomal DNA into nucleosomal units (Wyllie, 1980). On the other hand, necrosis is accompanied by the swelling of cells and organelles, in addition to the ultimate disintegration of the plasma membrane.

Apoptosis can be triggered by a variety of stimuli, including genotoxic agents, factor deprivation, and death factors (Nagata, 1997; Ashkenazi and Dixit, 1998; Green and Reed, 1998; Raff, 1998). It is involved in the removal of surplus cells generated during mammalian development, and in the removal of virally infected or cancer cells as part of the immune response (Raff et al., 1993; Nagata, 1997). Apoptotic signal transduction has been extensively studied biochemically and genetically, and it

is now known that a family of cysteine proteases with aspartate specificity are the major effectors of the process (Thornberry and Lazebnik, 1998). Caspases exist as inactive precursors in proliferating cells and are activated in a cascade. Upon exposure to apoptotic stimuli, initiator caspases are activated by the formation of a complex with other molecules. Once the initiator caspases are activated, they cause the processing of downstream caspases, which cleave a set of cellular proteins, leading to morphological changes and the degradation of chromosomal DNA. Necrosis is known to occur as a result of complement attack, severe hypoxia, hyperthermia, lytic viral infection, or exposure to a variety of toxins and respiratory poisons. Tumor necrosis factor (TNF)¹ can activate the necrotic death program in some cell lines, such as mouse L929 cells (Vercammen et al., 1998a). An involvement of necrosis in the removal of interdigital cells in the mouse embryo has also been suggested (Chautan

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¹Abbreviations used in this paper: BHA, butylated hydroxyanisole; DD, death domain; DED, death effector domain; FADD, Fas-associated protein with death domain; FasL, Fas ligand; FKBP, FK506-binding protein; LZ-FasL, FasL tagged with a leucine-zipper motif; PDTC, pyrrolidine dithiocarbamate; TNF, tumor necrosis factor; z-VAD-fmk, carbobenzoxy-Val-Ala-Asp-fluoromethylketone; $\Delta\Psi_m$, mitochondrial transmembrane potential.

et al., 1999). In contrast to apoptotic cell death, the molecular mechanism of necrosis is not well understood, though several mechanisms, including the release of lysosomal enzymes, the generation of toxic oxygen radicals, and the activation of calcium-dependent phospholipases, have been proposed (Fiers et al., 1999).

Fas ligand (FasL) is a member of the TNF family (Nagata and Golstein, 1995; Nagata, 1997) and was originally identified as a cytokine that triggers apoptosis by binding to Fas (Suda et al., 1993). Studies on FasL-induced apoptosis have revealed the following mechanism. The binding of FasL or agonistic anti-Fas antibody to Fas rapidly recruits procaspase 8 through an adaptor called FADD (Fas-associated protein with death domain) (Boldin et al., 1995; Chinnaiyan et al., 1995). FADD carries a domain called a death domain (DD) at its COOH terminus, which is involved in the binding to Fas. The NH₂-terminal part of FADD is called the death effector domain (DED) and is responsible for recruiting procaspase 8 (Boldin et al., 1996; Muzio et al., 1996). Thus, procaspase 8 oligomerized at the plasma membrane is autoactivated to a mature enzyme (Muzio et al., 1998; Yang et al., 1998). Two pathways have been shown for the signal transduction downstream of caspase 8, which are used in different cell types (types I and II) (Scaffidi et al., 1998). In type I cells, caspase 8 directly activates procaspase 3. However, in type II cells, caspase 8 cleaves Bid, a proapoptotic member of the Bcl-2 family (Li et al., 1998; Luo et al., 1998; Gross et al., 1999). The cleaved Bid translocates to the mitochondria and stimulates the release of cytochrome c. Cytochrome c, together with Apaf-1, activates procaspase 9 (Li et al., 1997), which leads to the downstream processing of procaspase 3.

We and others have recently established sublines from human Jurkat cells that do not express caspase 8 (Juo et al., 1998; Kawahara et al., 1998). Engagement of Fas by agonistic anti-human Fas antibodies in these cell lines does not activate caspases, and, thus, cannot induce apoptotic cell death. On the other hand, when FADD was artificially oligomerized, these cells died. This cell death process was not accompanied by either activation of the caspase cascade or apoptotic morphological cell changes. Rather, the cells showed a necrotic morphology, suggesting that FADD can mediate not only caspase-dependent apoptotic signals, but also caspase-independent necrotic death signals.

To examine the necrotic death-signaling pathway, we prepared various mutants of FADD and showed that FADD's DED domain is responsible for transducing the signal. This death process was accompanied by a loss of mitochondrial membrane potential, but not by the release of cytochrome c from mitochondria, and was inhibited by pyrrolidine dithiocarbamate (PDTC). We then showed that Fas engaged by FasL or anti-Fas antibodies could activate both caspase-dependent and -independent cell death signals. The caspase-dependent signal was rapid and was accompanied by the release of cytochrome c from mitochondria, as well as apoptotic morphological changes. In contrast, the caspase-independent cell death occurred slowly and was accompanied by the loss of mitochondrial membrane potential and necrotic morphological changes.

Materials and Methods

Cell Lines and Materials

Jurkat, a human T cell lymphoma cell line, and its derivative, JB6, which is deficient in caspase 8 (Kawahara et al., 1998), were maintained in RPMI 1640 medium containing 10% FCS (EQUITECH-BIO, Inc.). AP1510, a dimerizer of FK506-binding protein (FKBP) (Amara et al., 1997), was provided by Dr. M. Gilman (ARIAD Pharmaceuticals, Inc., Cambridge, MA). PDTC and carbobenzoyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) were purchased from Sigma-Aldrich and Takara Shuzo, Co., respectively. Monoclonal antibodies against human caspase 3 and FADD were purchased from Transduction Laboratories. The monoclonal antibodies against human cytochrome c, human cytochrome oxidase subunit II, and the FLAG epitope were from BD PharMingen, Molecular Probes, and Sigma-Aldrich, respectively. Horseradish peroxidase-conjugated anti-mouse IgG used as a secondary antibody was obtained from Dako. The recombinant human FasL tagged with a leucine-zipper motif (LZ-FasL) (Walczak et al., 1999) was produced by transfecting COS cells with the FasL-expression plasmid. The concentration of FasL in the supernatant of the transfected COS cells was determined by ELISA using anti-human FasL antibodies, as described previously (Tanaka et al., 1996).

Establishment of Stable Transformants

The plasmid pEF-FK-FADD carries the coding sequence (FKBP-FADD) for a myristilation-targeting peptide, two tandem repeats of FKBP12, and human FADD in the pEF-BOS mammalian expression vector (Kawahara et al., 1998). The DNA fragments coding for the DED of FADD (FADD-DED, amino acids 1–117), its truncated form (FADD- Δ DED, amino acids 79–117), and the DD of FADD (FADD-DD, amino acids 105–208) were prepared from human FADD cDNA (Boldin et al., 1995; Chinnaiyan et al., 1995). They were fused to a DNA fragment carrying a myristilation-targeting peptide and two tandem repeats of FKBP12, and inserted into pEF-BOS to generate pEF-FK-DED, pEF-F-FK-DED, pEF-F-FK- Δ DED, and pEF-FK-DD (Fig. 1 A). In pEF-F-FK-DED and pEF-F-FK- Δ DED, a DNA fragment coding for the FLAG epitope (DYKDDDDK) was inserted between the myristilation-targeting peptide and FKBP.

The stable cell transformants expressing FADD-DD, FADD-DED, and FADD- Δ DED were established, as previously described (Kawahara et al., 1998). In brief, cells were cotransfected with 50 μ g of the respective expression vectors with 1 μ g of the hygromycin-resistant gene (pMiwihy) by electroporation. Cells were cultured in the presence of 0.8–1.0 mg/ml of hygromycin, and the transformant clones expressing FADD-DD, FADD-DED, or FADD- Δ DED were identified by Western blotting using anti-FADD or anti-FLAG antibodies. Jurkat cell transformants expressing high levels of mouse Fas were established by introducing the mouse Fas expression plasmid (Watanabe-Fukunaga et al., 1992).

Cell Viability Assay and FACS[®] Analysis

Cell viability was determined by the WST-1 assay, as described previously (Kawahara et al., 1998). In brief, Jurkat and JB6 cell transformants (5×10^4 cells in 100 μ l) expressing the chimeric proteins, which were made from FADD and FKBP, were treated at 37°C with AP1510. Human Jurkat and its transformant expressing mouse Fas (JmF) were treated with 50 ng/ml LZ-FasL and 1 μ g/ml of the Jo2 antibody (Ogasawara et al., 1993), respectively. WST-1 reagents, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (Dojin Laboratories) and 1-methoxy-5-methylphenazinium methylsulfate (Dojin Laboratories), were added to the cells at final concentrations of 5.0 and 0.2 mM, respectively, and incubated for 1 h at 37°C. Using an ELISA autoreader, the cell viability was determined by measuring the difference between the absorbance at 450 and 620 nm. To examine the effect of various metabolic inhibitors on the cell death, cells were preincubated for 1 h with PDTC, z-VAD-fmk, or both.

The $\Delta\Psi_m$ and the formation of oxygen radicals were determined by staining cells with the Mitotracker orange reagents, CMTMRos, and CM-H₂TMRos (Molecular Probes), respectively, as described previously (Bossy-Wetzell et al., 1998). In brief, cells (10^6 cells/ml or otherwise stated) were incubated at 37°C for 30 min with 25 nM CMTMRos or 100 nM CM-H₂TMRos in RPMI1640 containing 10% FCS. Cells were washed with PBS and analyzed on a FACS[®]Calibur flow cytometer (Becton Dickinson) with excitation at 488 nm, using an argon laser. The fluorescence from CMTMRos was detected with a 585/42 nm band pass filter.

Subcellular Fractionation and Western Blotting

The subcellular fractions were prepared essentially according to Gross et al. (1999). In brief, 2×10^7 cells were washed with PBS, suspended in 150 μ l ice-cold isotonic homogenizing buffer (buffer A, 200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM Hepes-KOH, pH 7.4, 0.1 mM *p*APMSF), and homogenized in a glass Dounce homogenizer (Wheaton) with a tight pestle (10 strokes). Unlysed cells and nuclei were removed by centrifugation at 120 *g* for 5 min. The supernatant was spun at 10,000 *g* for 10 min, and the pellet, which was resuspended in buffer A, was used as the heavy membrane fraction containing mitochondria. The supernatant was further spun at 100,000 *g* for 30 min, and the resultant supernatant was used as the S100 fraction.

For Western blotting analysis, samples were mixed with an equal volume of $2\times$ Laemmli's sample buffer. After heating at 95°C for 10 min, proteins were separated by electrophoresis in a 15–25% gradient polyacrylamide gel (Dai-ichi Chemical), and then they were transferred to a polyvinylidene difluoride membrane (Hybond P; Amersham-Pharmacia

Biotech). The membrane was blocked with PBST (PBS supplemented with 0.05% Tween 20) containing 5% nonfat dry milk, followed by successive incubations with primary and secondary antibodies. Proteins were visualized with the enhanced chemiluminescence system (Renaissance; NEN Life Science Products Inc.).

Results

Requirement of DED for Necrotic Death Induced by FADD Oligomerization

Artificial oligomerization of FADD can kill JB6 cells; this death is accompanied by necrotic morphological changes and occurs in a caspase-independent manner (Kawahara et al., 1998). FADD contains two distinct domains, the DD and DED (Boldin et al., 1995; Chinnaiyan et al., 1995), of which DED mediates the apoptotic cell death by recruiting caspase 8. To examine which domain is responsible for the caspase-independent cell death process, FADD-DD or FADD-DED was joined to two copies of FKBP, and the resulting expression plasmids were introduced into Jurkat or JB6 cells (Fig. 1 A). When the transformants were treated for 6 h with AP1510 to oligomerize the chimeric proteins, Jurkat cell transformants expressing FKBP-DED, but not FKBP-DD, were killed in a dose-dependent manner (Fig. 1 B). Oligomerization of FKBP-DED, but not FKBP-DD, also efficiently killed the JB6 cells. When the DED was truncated, its death-inducing activity was destroyed. As found with FKBP-FADD (Kawahara et al., 1998), the FKBP-induced death of Jurkat cells was accompanied by caspase activation and apoptotic morphological changes. In contrast, the oligomerization of FKBP-DED in JB6 cells did not activate caspases, though it did induced necrotic morphological changes (data not shown). These results indicate that the FADD-DED is responsible for transducing not only the caspase-mediated apoptotic signal, but also the caspase-independent death signal.

Inhibitory Effect of PDTC on Caspase-independent Cell Death

To study the molecular mechanism of the caspase-independent necrotic pathway, we first screened various compounds for the ability to inhibit the FKBP-FADD-induced death of JB6 cells. We found that PDTC inhibits the process in a dose-dependent manner. As shown in Fig. 2 A, AP1510 treatment killed 90% of the cells within 4 h. However, when the cells were preincubated with 80 μ M PDTC, 90% of the cells survived for at least 4 h. A similar inhibitory effect of PDTC was observed with JB6 cells expressing FKBP-DED. That is, death was substantially delayed by pretreating the cells with PDTC (Fig. 2 B). PDTC is known to work as an antioxidant (Liu et al., 1996). However, other antioxidants, such as butylated hydroxyanisole (BHA, 250 μ M) and nordihydroguaiaretic acid (25 μ M), showed little inhibitory effect on the FADD-induced death of JB6 cells (data not shown).

Changes of Mitochondrial Membrane Potential and Release of Cytochrome *c* in Caspase-independent Cell Death

The death of JB6 cells induced by the oligomerization of FADD is accompanied by massive swelling of the mito-

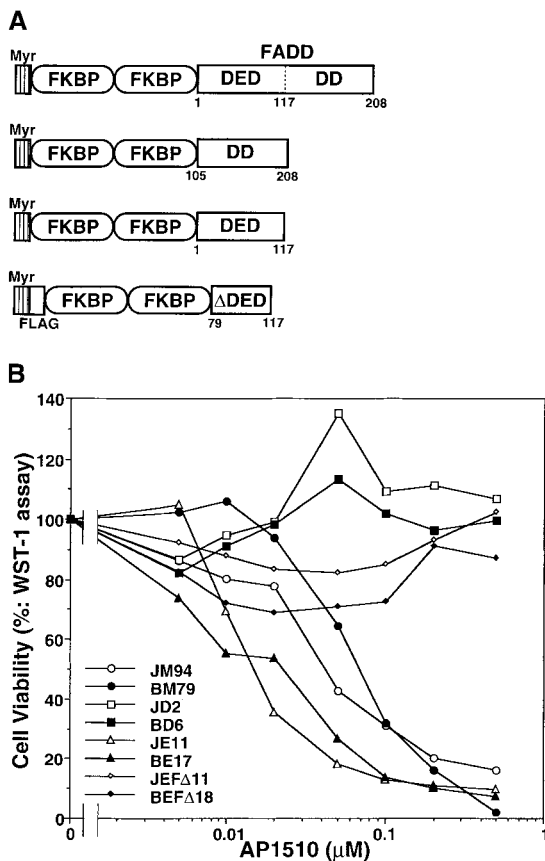


Figure 1. Requirement for the DED in FADD-mediated and caspase-independent cell death. (A) Structure of chimeric molecules of FKBP with FADD and its derivatives. FKBP represents the region of amino acids 2–108 of human FK506-binding protein. FADD-DD, FADD-DED, and FADD- Δ DED contain amino acids 105–208, 1–117, and 79–117 of human FADD, respectively. Myr and FLAG indicate the myristylation-targeting peptide and FLAG epitope, respectively. (B) Death of Jurkat and JB6 cells by oligomerization of FADD-DED. Jurkat (open symbols) and JB6 (closed symbols) cells were transformed with the expression plasmid for FADD (circles), FADD-DD (squares), FADD-DED (triangles), or FADD- Δ DED (diamonds). The stable transformant clones were incubated at 37°C for 6 h with the indicated concentrations of AP1510, and the cell viability was determined by the WST-1 method. The results are expressed as a percent of the value obtained without AP1510. The experiments were performed in triplicate, and the average values were plotted.

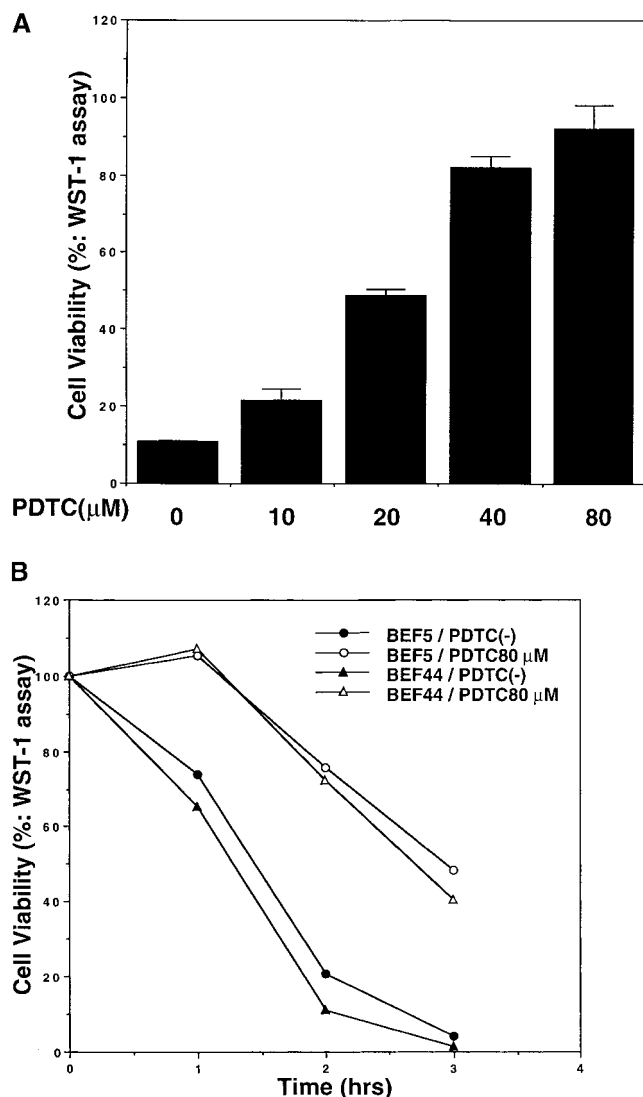


Figure 2. Inhibitory effect of PDTC on the cell death induced by FADD oligomerization. (A) Dose-dependent effect of PDTC. JB6 cell transformants expressing FKBP-FADD were pretreated at 37°C for 1 h with the indicated concentrations of PDTC. The cells were divided into two samples, and each was incubated at 37°C for 4 h, with or without 0.5 μM AP1510. The WST-1 assay was carried out as described in Materials and Methods, and the cell viability of the PDTC-treated cells is expressed as the percent of the value obtained without AP1510. The experiments were performed in triplicate, and the average values were plotted with SD (bars). (B) Time-dependent cell death by FADD oligomerization. Two JB6 transformant clones (circles and triangles) expressing FKBP-DED were preincubated at 37°C for 1 h, with (open symbols) or without (closed symbols) 80 μM PDTC. The cells were then incubated for the indicated time periods with 0.5 μM AP1510, and the cell viability was determined as described above.

chondria (Kawahara et al., 1998). To examine whether mitochondria are functional in JB6 cells that are dying due to the oligomerization of DED, the $\Delta\Psi_m$ was measured by staining with the Mitotracker reagent. As shown in Fig. 3 A, $\Delta\Psi_m$ dramatically decreased within 3 h after the addition of AP1510. This reduction in $\Delta\Psi_m$ was greatly inhibited by preincubation of the cells with 80 μM PDTC, which is consistent with the inhibitory effect of PDTC on cell death. The TNF-induced necrotic cell death of mouse

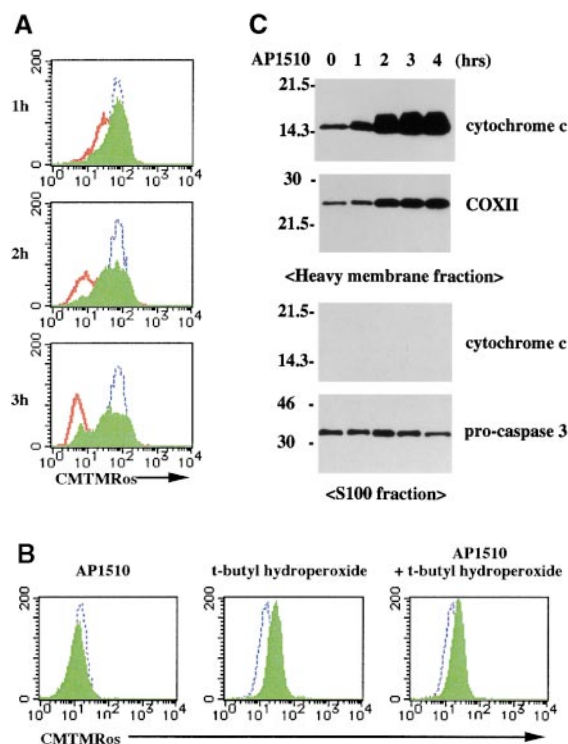


Figure 3. Changes of mitochondrial functions in the FADD-DED-induced death of JB6 cells. (A) Reduction of $\Delta\Psi_m$ by FADD oligomerization. JB6 cell transformants expressing FKBP-DED were preincubated for 1 h, with (green areas) or without (red lines) 80 μM PDTC, and then were incubated at 37°C, for the indicated time periods, with 0.5 μM AP1510. The $\Delta\Psi_m$ was determined by the Mitotracker orange reagent CMTMRos, as described in Materials and Methods. The FACS[®] profiles of the cells without treatment with AP1510 are shown in open areas with dotted lines. (B) No oxygen radical formation in mitochondria by FADD oligomerization. JB6 cell transformants expressing FKBP-DED were incubated at 37°C for 1 h with 0.5 μM AP1510, 0.73 mM *t*-butyl hydroperoxide, or both. The cells were then stained with the Mitotracker orange reagent CM-H₂TMRos, and the FACS[®] profiles are shown by the green areas. The open areas show the FACS[®] profiles for the proliferating cells. (C) No release of cytochrome c from mitochondria during FADD-DED-induced death of JB6 cells. A JB6 transformant clone expressing FKBP-DED was incubated at 37°C for the indicated time periods with 0.5 μM AP1510. The cells were homogenized with a Dounce homogenizer, and the cell extracts were fractionated into heavy membrane and soluble fractions by centrifugation, as described in Materials and Methods. Proteins (5 μg) of the heavy membrane and S100 fraction were separated by SDS-PAGE, blotted onto a PVDF membrane, and probed with monoclonal antibodies against cytochrome c, cytochrome oxidase subunit II (COXII), and human caspase 3. Sizes of molecular weight standard proteins are shown in kilodaltons to the left.

L929 cells is accompanied by formation of oxygen radicals (Vercammen et al., 1998a). To examine whether oxygen radicals are involved in the loss of $\Delta\Psi_m$ of FADD-DED-induced death of JB6 cells, the cells were stained with a reduced version of the Mitotracker reagent (CM-H₂TMRos) that emits fluorescence only after oxidation. As shown in Fig. 3 B, the staining intensity with this reagent did not increase after the treatment with AP1510. This was not due to the inability of the reagent to enter mitochondria, because the treatment of the cells with *t*-butyl hydroperoxide

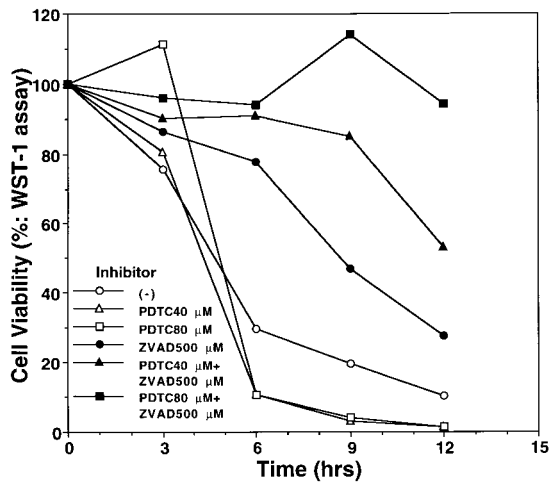


Figure 4. Inhibitory effects of a caspase inhibitor and PDTC on the Fas-induced cell death. JmF cells were incubated with 1 μ g/ml Jo2 antibody alone (\circ) or in the presence of 40 μ M PDTC (Δ), 80 μ M PDTC (\square), 0.5 mM z-VAD-fmk (\bullet), 40 μ M PDTC and 0.5 mM z-VAD-fmk (\blacktriangle), and 80 μ M PDTC and 0.5 mM z-VAD-fmk (\blacksquare). After incubation at 37°C for the indicated time periods, cell viability was determined by the WST-1 assay, as described in Materials and Methods. The results are expressed as the percent of the value obtained without the Jo-2 antibody. The experiments were performed in triplicate, and the average values were plotted.

significantly increased the fluorescence in the presence or absence of AP1510.

Various apoptotic stimuli cause the release of cytochrome c from mitochondria (Kluck et al., 1997; Yang et al., 1997), and this release had been proposed to be due to the loss of $\Delta\Psi_m$ (Susin et al., 1997). Therefore, we examined whether cytochrome c was released from mitochondria during the DED-induced death of JB6 cells. The JB6 cells expressing FKBP-DED were treated with AP1510 for various lengths of time and homogenized with a Dounce homogenizer. After removing the unlysed cells, the cell extracts were fractionated into heavy membrane (precipitates of 10,000 g) and cytosolic (supernatant of 100,000 g) fractions, and then they were analyzed by Western blotting with anti-cytochrome c antibodies. As shown

in Fig. 3 C, no cytochrome c was detected in the S100 fraction, even 4 h after the addition of AP1510. Rather, the apparent content of cytochrome c in the heavy membrane fractions increased. The amount of cytochrome oxidase subunit II in this fraction increased similarly in a time-dependent manner. These results may indicate that the relative proportion of mitochondria increased in the heavy membrane fraction because the dying cells were more easily disrupted. Cytochrome c that is released into the cytosol can process procaspase 3 into its mature form through the activation of caspase 9 (Li et al., 1997). In agreement with the observed lack of cytochrome c release from mitochondria, the procaspase 3 remained intact 4 h after the addition of AP1510 (Fig. 3 C).

The Death Signals from the Fas Receptor in Fas-overexpressing Cells

When the endogenously expressed Fas of Jurkat cells is engaged by anti-human Fas antibodies, the cells die, and this process can be blocked by caspase inhibitors. On the other hand, the above results indicated that JB6 cells, a Jurkat-derived cell line, can be killed in a caspase-independent manner when FADD or its DED is tightly aggregated. To examine whether the caspase-independent death signal can be transduced from Fas, Jurkat cell transformants (JmF) that overexpress mouse Fas were established. As shown in Fig. 4, when the transformants were treated with the anti-mouse Fas antibody (Jo2), the cells died. This cell death process was quicker than that observed with JmF cells treated with the anti-human Fas antibody, suggesting that overexpression of mouse Fas and its engagement with the anti-mouse Fas antibody can generate a stronger death signal than that elicited through endogenously expressed Fas. Preincubation of JmF cells with 0.5 mM z-VAD-fmk, but not with PDTC, significantly blocked the anti-mouse Fas-induced death process (Fig. 4). However, PDTC in the presence of z-VAD-fmk had an additional inhibitory effect on the death process in a dose-dependent manner. Thus, preincubation of the cells with 0.5 mM z-VAD-fmk and 80 μ M PDTC almost completely inhibited the Fas-induced death of JmF cells. These results indicate that when the caspases are functional, the caspase-dependent death pathway dominates the caspase-independent pathway. How-

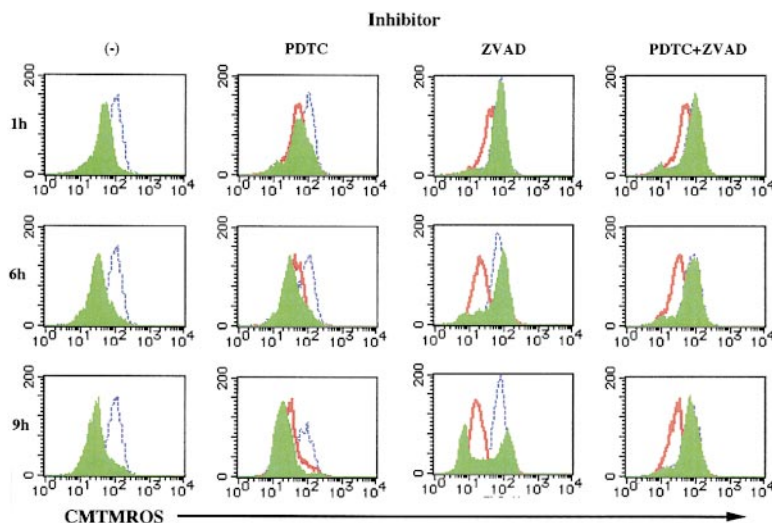


Figure 5. Inhibitory effects of PDTC and z-VAD-fmk on Fas-induced reduction of the $\Delta\Psi_m$. JmF cells (10^6 cells/ml) were incubated at 37°C with 1 μ g/ml Jo2 antibody alone (first column) or in the presence of 80 μ M PDTC (second column), 0.5 mM z-VAD-fmk (third column), or 80 μ M PDTC and 0.5 mM z-VAD-fmk (fourth column). After incubation for the indicated time periods (shown to the left of each row), $\Delta\Psi_m$ was measured, as described in Materials and Methods. The FACS[®] profiles are shown in the green areas. The FACS[®] profiles of cells without anti-Fas antibody treatment are indicated by open areas with dotted lines in all panels, whereas those obtained without inhibitors are indicated by open areas with red lines in the panels of the second, third, and fourth columns.

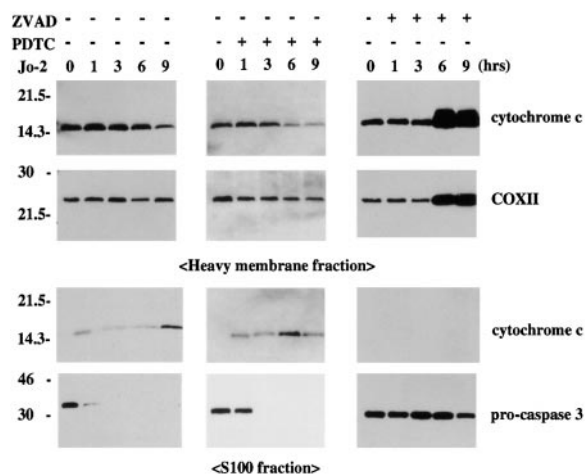


Figure 6. Selective inhibition of the Fas-induced apoptotic pathway by a caspase inhibitor. JmF cells were incubated with 1 $\mu\text{g/ml}$ Jo2 antibody at 37°C for the indicated time periods in the absence (–) or presence (+) of 0.5 mM z-VAD–fmk or 80 μM PDTC. Cells were homogenized with a Dounce homogenizer, and the heavy membrane and S100 fractions were prepared by centrifugation. Proteins (5 μg) of the heavy membrane and S100 fractions were separated by SDS-PAGE and subjected to Western Blotting using monoclonal antibodies against cytochrome c, cytochrome oxidase subunit II (COXII), and caspase 3. Sizes of molecular weight standards are shown in kilodaltons to the left.

ever, even when the caspases are not functional, cells die by Fas activation via a caspase-independent mechanism.

The change in $\Delta\Psi\text{m}$ in the Fas-activated cells was then measured in the absence or presence of z-VAD–fmk and PDTC. As shown in Fig. 5, Fas activation caused a gradual reduction in the $\Delta\Psi\text{m}$ of JmF cells. PDTC had little effect on the kinetics of the $\Delta\Psi\text{m}$ loss. Preincubation of the cells with z-VAD–fmk blocked the loss of $\Delta\Psi\text{m}$ at an early stage of cell death. That is, >80% of the cells showed an intact $\Delta\Psi\text{m}$ 6 h after Fas activation, which agrees with the scenario that most cells were alive at this point (Fig. 4). However, at a later time point (9 h after the addition of anti-Fas antibody), the $\Delta\Psi\text{m}$ of half the cell population was com-

pletely lost. This loss of $\Delta\Psi\text{m}$ was blocked by adding PDTC together with z-VAD–fmk. These results suggest that the gradual reduction in $\Delta\Psi\text{m}$ during the Fas-induced cell death process is caspase dependent, whereas the Fas-induced caspase-independent death signal causes a sharper reduction in $\Delta\Psi\text{m}$ that occurs later in the cell-death process.

We then examined the release of cytochrome c from mitochondria during the Fas-activated death of JmF cells. As shown in Fig. 6, cytochrome c was detected in the cytosolic fraction within 1 h of Fas activation, which was accompanied by the processing of procaspase 3. Pretreatment of the cells with PDTC had no effect on the release of cytochrome c, though it slightly delayed the processing of procaspase 3. In contrast, z-VAD–fmk completely blocked the release of cytochrome c, indicating that the release of cytochrome c from mitochondria is a caspase-dependent process. The amount of mitochondria in the heavy membrane fractions was also enhanced under these conditions.

Apoptotic and Necrotic Morphological Changes in the Fas-activated JmF Cells

The above results suggested that Fas can transduce the caspase-dependent as well as -independent cell death signals. To correlate of these cell death processes with apoptosis and necrosis, the Fas-activated dying cells were examined by transmission electron microscopy. As previously reported with various Fas-expressing cells (Itoh et al., 1991; Ni et al., 1994), the engagement of Fas by the agonistic anti-Fas antibody in JmF cells caused condensation and fragmentation of the nuclei, as well as shrinkage of the cells (Fig. 7), which are characteristics of apoptosis. These apoptotic morphological changes were not inhibited by preincubation with PDTC. When the cells were stimulated with the anti-Fas antibody in the presence of z-VAD–fmk, the cells looked morphologically normal for up to 6 h (data not shown). However, at 9 h after the Fas activation, their cytoplasm looked electron translucent and their mitochondria showed a high degree of swelling, characteristics that accompany necrotic cell death. The addition of PDTC together with z-VAD–fmk blocked the bleaching of the cytoplasm and the swelling of the mitochondria, though many vacuole-like structures were observed in the cells.

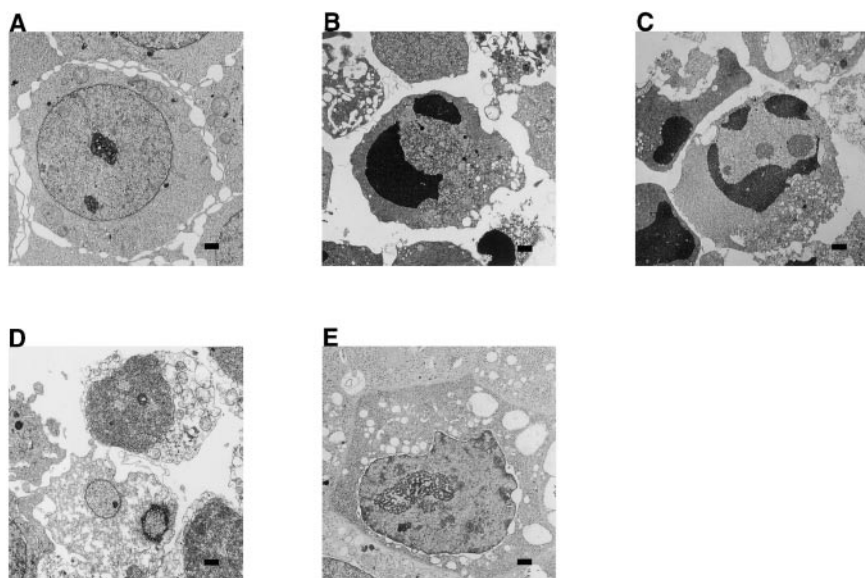


Figure 7. Apoptotic and necrotic cell death through the Fas receptor. JmF cells were preincubated at 37°C for 1 h with 80 μM PDTC (C), 0.5 mM z-VAD–fmk (D), or 80 μM PDTC plus 0.5 mM z-VAD–fmk (E). Then the cells were treated at 37°C for 12 h with 1 $\mu\text{g/ml}$ Jo2 antibody and examined by electron transmission microscopy using a Hitachi H7100. As controls, cells were left untreated (A) or treated with Jo2 antibody without preincubation with inhibitors (B). Bars, 1 μm .

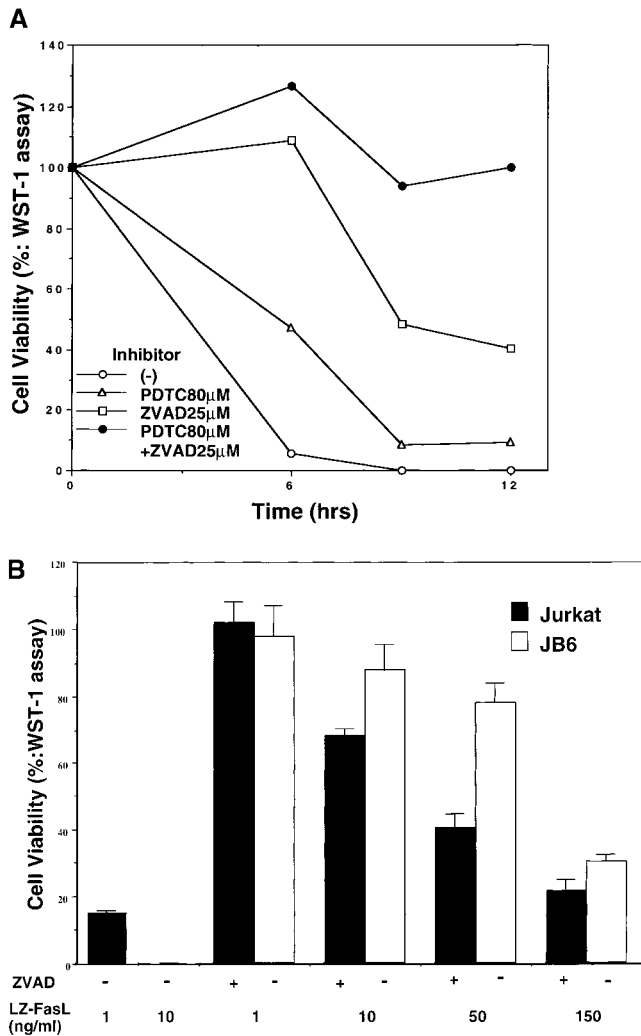


Figure 8. Effects of z-VAD-fmk and PDTC on the FasL-induced cell death of Jurkat cells. (A) Effects of inhibitors on the cell death. Jurkat cells were incubated with 50 ng/ml LZ-FasL alone (○) or in the presence of 80 μ M PDTC (△), 25 μ M z-VAD-fmk (□), or 80 μ M PDTC plus 25 μ M z-VAD-fmk (●). After incubation at 37°C for the indicated time periods, cell viability was determined by the WST-1 assay, as described in Materials and Methods. The results are expressed as the percent of the value obtained without LZ-FasL. The experiments were performed in triplicate, and the average values were plotted. (B) A dose-dependent killing of JB6 cells by LZ-FasL. Jurkat cells (black bars) and JB6 cells (white bars) were incubated at 37°C for 12 h with the indicated concentration of LZ-FasL. The Jurkat cells were treated with LZ-FasL in the absence (-) or presence of 25 μ M z-VAD-fmk (+). The experiments were carried out in triplicate, and the average values were shown with SD. Similar results were obtained by two other independent experiments.

The FasL-Induced Death Signals in Human Jurkat Cells

The above results indicated that the activation of Fas can activate both apoptotic and necrotic signals, at least in Fas-overexpressing Jurkat cells. Under physiological conditions, FasL binds to the Fas receptor and activates the death signals. FasL is a type II membrane protein, and the cell-associated FasL has a stronger cytotoxic activity than its soluble form (Tanaka et al., 1998). Recently, Walczak et al. (1999) prepared the soluble form of FasL, which was

tagged with a leucine-zipper motif, and showed that it has a strong cytotoxic activity. In fact, wild-type Jurkat cells were efficiently killed by the treatment with LZ-FasL. As shown in Fig. 8 A, LZ-FasL, at 50 ng/ml, killed most of Jurkat cells within 6 h, which is much faster than that observed with anti-human Fas monoclonal antibody (CH-11) (Kawahara et al., 1998). Although this cell death process was inhibited by the addition of 25 μ M of z-VAD-fmk at its earlier stage (6 h), cells still died when they were incubated for >9 h (Fig. 8 A). This cell death was blocked by adding PDTC, suggesting that FasL can activate caspase-dependent and -independent cell deaths. We then examined whether JB-6 cells were sensitive to LZ-FasL-induced cell death. As shown in Fig. 8 B, JB-6 cells lacking caspase 8 were killed within 12 h in a dose-dependent manner. The concentration of LZ-FasL required to give a half-maximal killing was slightly higher than that required to kill Jurkat cells in the presence of z-VAD (Fig. 8 B), but a similar concentration of the supernatant of COS cell, which were transfected with the empty vector, had no effect (data not shown). These results indicate that FasL, the physiological ligand for Fas, can activate caspase-dependent and -independent cell death.

Discussion

Apoptosis and necrosis have traditionally been regarded as two different death processes induced by different stimuli (Wyllie et al., 1980; Duval and Wyllie, 1986). However, recent studies indicate that some stimuli, such as TNF, can induce either apoptosis or necrosis, depending on the cell type (Vercammen et al., 1998a; Fiers et al., 1999). Genotoxic agents that cause apoptosis also cause necrosis in several cell lines when the apoptotic process is inhibited by caspase inhibitors (Lemaire et al., 1998). We showed here that Fas can transduce caspase-dependent apoptotic and caspase-independent necrotic death signals. Both signals were mediated through the DED of FADD. In JmF cells, the apoptotic pathway dominated the necrotic pathway at the early stage and was accompanied by the release of cytochrome c from mitochondria and the processing of procaspase 3. According to Scaffidi et al. (1998), human Jurkat cells are the type II cells in which caspase 8 that is activated by Fas engagement cleaves Bid and causes cytochrome c release from the mitochondria (Krammer, 1999). The apoptotic pathway, including the cytochrome c release in JmF cells, was inhibited by a broad caspase inhibitor, z-VAD-fmk, a result that agrees with the model for type II cells. The loss of mitochondrial membrane potential ($\Delta\Psi_m$) is observed in the apoptosis induced by a variety of stimuli (Shimizu et al., 1996; Zamzami et al., 1996) and has been proposed to be essential for the release of cytochrome c and/or apoptosis-inducing factor from mitochondria (Susin et al., 1997). However, even when $\Delta\Psi_m$ was substantially lost during the necrosis of JmF cells, no release of cytochrome c was observed (Fig. 6), suggesting that the loss of $\Delta\Psi_m$ is not sufficient to release cytochrome c from mitochondria. A similar conclusion was reported by Bossy-Wetzel et al. (1998) for the staurosporine-induced cell death. A slight and gradual loss of $\Delta\Psi_m$ during the Fas-induced apoptosis of JmF cells may be a secondary effect caused by the release of cytochrome c.

In contrast to apoptosis, the necrotic pathway functioned independent of the caspases. Even caspase 8-null

cells died when FADD was oligomerized. This process was accompanied by a sudden loss of $\Delta\Psi_m$. Such a loss of $\Delta\Psi_m$ would reduce the cellular ATP level, which may lead to necrotic cell death (Eguchi et al., 1997; Leist et al., 1997). In mouse L929 cells, an excess of oxygen radicals is produced upon activation with TNF and Fas (Vercammen et al., 1998a, b). Since BHA, a radical scavenger, blocks the TNF- and Fas-induced necrotic pathway in L929 cells, it was postulated that oxygen radicals are responsible for necrotic cell death. Although reactive oxygens would reduce the $\Delta\Psi_m$, we could not detect a significant production of reactive oxygen intermediates during the FADD-DED-induced necrosis in JB6 cells, and BHA could not inhibit the death process, suggesting that oxygen radicals are not responsible for the loss of $\Delta\Psi_m$ in our Jurkat system. In contrast, we found that PDTC efficiently inhibited Fas- or FADD-DED-induced necrosis. How PDTC blocks the process is not clear. PDTC is a thiol compound, works as an antioxidant or prooxidant, and has cytotoxic effects (Orrenius et al., 1996). It inhibits the induction of some transcription factors such as NF- κ B, whereas it activates other transcription factors such as AP-1, p53, and NF-AT. Although the JNK-AP1 pathway has been suggested for Fas-mediated cell death, we found no significant activation of JNK in FADD-induced necrotic cell death (Shimizu, Y., H. Matsumura, A. Kawahara, and S. Nagata, unpublished observations). Moreover, inhibitors of protein synthesis or RNA synthesis (cycloheximide or actinomycin D) did not inhibit the FADD-DED-mediated necrosis (data not shown), indicating that, as for Fas-mediated apoptosis, all components necessary for necrotic cell death are present in proliferating cells. It is likely that a DED-containing molecule(s) binds to FADD-DED and is activated by oligomerization. In any case, this signaling pathway should lead to the loss of $\Delta\Psi_m$ and should be inhibited by PDTC. Identification of molecules involved in necrotic signal transduction and the development of its specific inhibitor will be necessary to address whether or not the inhibition of loss of $\Delta\Psi_m$ blocks eventual cell death.

Our present results indicate that Fas can activate two distinct death pathways that branch at FADD. Since embryonal fibroblasts from caspase 8-null mice or caspase 8-deficient Jurkat cells are resistant to the Fas-activated death (Kawahara et al., 1998; Varfolomeev et al., 1998), we think that the engagement of Fas usually causes apoptosis. However, when Fas is strongly activated for long periods of time without phagocytosis of the apoptotic cells, the cells may undergo necrotic cell death. In this regard, it is noteworthy that the administration of FasL or agonistic anti-Fas antibodies to animals brings about massive hemorrhagic necrosis in the liver (Ogasawara et al., 1993; Walczak et al., 1999), suggesting that Fas can transduce necrotic death signal under some pathological conditions. Recently, Lauzurica et al. (1999) showed that TNF- α -induced septic shock in vivo can be blocked by pretreating mice with PDTC, suggesting that TNF α can also transduce the PDTC-inhibited death signal. The demonstration that FADD-DED is responsible for the necrotic death signal in caspase 8-null cells will assist further studies targeted at identifying other molecules involved in necrotic cell death.

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