Loss of Caspase-9 Reveals Its Essential Role for Caspase-2 Activation and Mitochondrial Membrane Depolarization

Ajoy K. Samraj, Dennis Sohn, Klaus Schulze-Osthoff,* and Ingo Schmitz*

Institute of Molecular Medicine, University of Düsseldorf, Düsseldorf D-40225, Germany

Submitted April 3, 2006; Revised September 27, 2006; Accepted October 25, 2006

Monitoring Editor: Gerard Evan

Caspase-9 plays an important role in apoptosis induced by genotoxic stress. Irradiation and anticancer drugs trigger mitochondrial outer membrane permeabilization, resulting in cytochrome c release and caspase-9 activation. Two highly contentious issues, however, remain: It is unclear whether the loss of the mitochondrial membrane potential $\Delta\Psi_M$ contributes to cytochrome c release and whether caspases are involved. Moreover, an unresolved question is whether caspase-2 functions as an initiator in genotoxic stress-induced apoptosis. In the present study, we have identified a mutant Jurkat T-cell line that is deficient in caspase-9 and resistant to apoptosis. Anticancer drugs, however, could activate proapoptotic Bcl-2 proteins and cytochrome c release, similarly as in caspase-9-proficient cells. Interestingly, despite these alterations, the cells retained $\Delta\Psi_M$. Furthermore, processing and enzyme activity of caspase-2 were not observed in the absence of caspase-9. Reconstitution of caspase-9 expression restored not only apoptosis but also the loss of $\Delta\Psi_M$ and caspase-2 activity. Thus, we provide genetic evidence that caspase-9 is indispensable for drug-induced apoptosis in cancer cells. Moreover, loss of $\Delta\Psi_M$ can be functionally separated from cytochrome c release. Caspase-9 is not only required for $\Delta\Psi_M$ loss but also for caspase-2 activation, suggesting that these two events are downstream of the apoptosome.

INTRODUCTION

Cell death can occur via distinct biochemical pathways and morphological alterations, one of which is apoptosis (Leist and Jaattela, 2001). Cell death by genotoxic drugs is usually mediated by the intrinsic mitochondrial apoptotic pathway, which is regulated by pro- and anti-apoptotic proteins of the Bcl-2 family (Cory *et al.*, 2003). Activation of the proapoptotic family members Bax and Bak leads to mitochondrial outer membrane permeabilization (MOMP) and release of apoptogenic factors from the mitochondrial intermembrane space, including cytochrome c (Wang, 2001).

Another characteristic event observed during cell death is the loss of the mitochondrial transmembrane potential $\Delta\Psi_M$, the electrochemical proton gradient generated by the respiratory chain (Newmeyer and Ferguson-Miller, 2003). It is currently unclear how the loss of $\Delta\Psi_M$ contributes to the apoptotic process. Breakdown of $\Delta\Psi_M$ could be caused by opening of the permeability transition pore, which is a large protein channel spanning the outer and inner mitochondrial membrane, during a process called mitochondrial permeability transition (Zamzami and Kroemer, 2001). A second model suggests that only Bcl-2 family proteins are necessary for MOMP and cytochrome c release and that the loss of $\Delta\Psi_M$ is a downstream and caspase-dependent phenomenon (Martinou and Green, 2001; Ricci *et al.*, 2004). Currently,

there is major controversy over whether the loss of $\Delta\Psi_M$ is coupled to and required for MOMP and cytochrome c release or whether loss of $\Delta\Psi_M$ is a downstream and caspase-dependent phenomenon.

Caspases are aspartate-specific cysteine proteases that are synthesized in cells as inactive zymogens containing a prodomain and a large and a small subunit (Fuentes-Prior and Salvesen, 2004). The active enzyme is composed of a heterotetramer formed by two large and two small subunits. Caspases can be divided functionally into initiator and executioner caspases (Fuentes-Prior and Salvesen, 2004). Initiator caspases, such as caspase-8, -9, and -10, are characterized by a long prodomain, containing protein-protein interaction motifs. These interaction motifs allow dimerization of the initiator caspases, which is sufficient for initial enzyme activity and autoproteolytic cleavage (Boatright *et al.*, 2003; Donepudi *et al.*, 2003; Baliga *et al.*, 2004). Activation of executioner caspases occurs by cleavage between the subunits. On processing, initiator caspases become fully active and activate downstream executioner caspases, such as caspase-3, -6, and -7, which then cleave key substrates, leading to apoptotic cell death (Fischer *et al.*, 2003).

The dimerization and activation of the initiator caspases occurs at multiprotein complexes. In death receptor pathway, caspases-8 and -10 are activated at a death-inducing signaling complex (DISC) formed upon ligand binding (Peter and Krammer, 2003). In the mitochondrial pathway caspase-9 serves as an initiator caspase. When cytochrome c is released from the mitochondrial intermembrane space, it binds to Apaf-1, leading to the recruitment and activation of caspase-9 in a high-molecular-weight complex called the apoptosome (Wang, 2001).

Similarly to caspase-8, -9, and -10, caspase-2 is characterized by a long prodomain and is thus often regarded as a bona fide initiator caspase. The cleavage specificity of caspase-2, however, is more related to effector caspases

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06-04-0263>) on November 1, 2006.

* These authors contributed equally to this work.

Address correspondence to: Ingo Schmitz (ingo-schmitz@uni-duesseldorf.de) or Klaus Schulze-Osthoff (kso@uni-duesseldorf.de).

Abbreviations used: $\Delta\Psi_M$, mitochondrial membrane potential; MOMP, mitochondrial outer membrane permeabilization; PTP, permeability transition pore.

(Thornberry *et al.*, 1997), making it difficult to assign a function of caspase-2 as a regulatory or downstream protease. Recent reports demonstrated that caspase-2 might act as an apical protease in stress- or death receptor-mediated apoptosis (Lassus *et al.*, 2002; Robertson *et al.*, 2002; Tinel and Tschoop, 2004; Wagner *et al.*, 2004; Werner *et al.*, 2004). Moreover, it was suggested that caspase-2 is required for MOMP and the release of cytochrome c in response to DNA-damaging agents (Guo *et al.*, 2002; Robertson *et al.*, 2002; Enoksson *et al.*, 2004). Other reports suggested that caspase-2 is activated downstream of Bax and Bak and cannot bypass the apoptosome (O'Reilly *et al.*, 2002; Ruiz-Vela *et al.*, 2005). Thus, there is conflicting evidence of whether caspase-2 functions as an initiator or effector during apoptosis. Moreover, elucidation of the functional role of caspase-2 has been obscured by the lack of an overt phenotype of caspase-2 knockout mice (Bergeron *et al.*, 1998; O'Reilly *et al.*, 2002).

Here we describe the identification of a caspase-9-deficient Jurkat clone that is resistant to induction of apoptosis by genotoxic agents and activates virtually no caspase in response to DNA damage. Analysis of mitochondrial events showed that caspase-9-deficient cells released cytochrome c upon DNA damage but, interestingly, retained their mitochondrial membrane potential $\Delta\Psi_M$. When caspase-9 was reconstituted, both apoptosis and loss of $\Delta\Psi_M$ were restored, clearly suggesting that cytochrome c release and $\Delta\Psi_M$ breakdown are separate and consecutive events, with the latter being caspase-dependent. Finally, we demonstrate that during genotoxic stress caspase-2 processing as well as acquisition of its catalytic activity is dependent on caspase-9.

MATERIALS AND METHODS

Cell Lines and Reagents

All cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U of penicillin/ml, and 0.1 mg streptomycin/ml (PAA Laboratories, Linz, Austria). Cells were grown at 37°C in a humidified 5% CO₂ atmosphere and maintained in the logarithmic phase. Anti-Bid, anti-caspase-3 mAb, and polyclonal anti-caspase-9 were purchased from R&D Systems (Wiesbaden, Germany) and New England BioLabs (Beverly, MA). Bax and cytochrome c monoclonal antibodies were obtained from PharMingen (Hamburg, Germany). The conformation-specific anti-Bak mAb was from Oncogene (La Jolla, CA). Monoclonal antibodies against β -actin and Bcl-2 as well as antisera against c-IAP1 and c-IAP2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Bim mAb was from Alexis (Grünwald, Germany) and polyclonal anti-Bcl-x_L from Transduction Laboratory (Heidelberg, Germany). The anti-caspase-8 mAb was obtained from Biocheck (Münster, Germany), and antibodies against caspase-2 were from Alexis and Santa Cruz Biotechnology. The anti-Apaf-1 polyclonal antibody was from Chemicon (Temecula, CA). Secondary antibodies, anti-mouse IgG, and anti-rabbit IgG coupled to horseradish peroxidase were purchased from Promega (Mannheim, Germany). Anti-goat IgG coupled to horseradish peroxidase was purchased from Molecular Probes (Karlsruhe, Germany) and biotin-VAD-fmk (biotin-Val-Ala-Asp-[OMe]-fluoromethylketone) from ICN (Eschwege, Germany). Staurosporine, etoposide, doxorubicin, daunorubicin, and propidium iodide (PI) were obtained from Sigma (Deisenhofen, Germany). CD95L was a gift of Dr. Harald Wajant.

Reverse Transcriptase-PCR

RNA was isolated from cells using a total RNA isolation kit (Qiagen, Hilden, Germany). Reverse transcriptase (RT) reaction and PCR were performed using the titanium one-step RT-PCR kit (BD Biosciences, Heidelberg, Germany). For standardization, each RT sample was PCR-amplified for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The 3' and 5' primers used for amplification were (5'-ATG GAC GAA GCG GAT CCG) and (5'-CCC TGG CCT TAT GAT GTT-3') for caspase-9, and (5'-GTG GAA GGA CTC ATG ACC ACA G-3') and (5'-CTG GTG CTC AGT GTA GCC CAG-3') for GAPDH.

Stable Expression of Caspase-9

Caspase-9-deficient Jurkat cells were electroporated with a GenePulser II (Bio-Rad, Munich, Germany) in a 0.4-cm cuvette at 250 V and 950 μ F with 20

μ g of DNA in 200 μ l per transfection and selected for G418 resistance. The N-terminally Flag-tagged procaspase-9 construct was kindly provided by G. Salvesen.

Flow Cytometric Analyses

Cells (1×10^6 per assay) were stimulated for the indicated time in a 24-well plate with 100 μ M etoposide, 1 μ M doxorubicin, 1 μ M daunorubicin, or 2.5 μ M staurosporine or were left untreated. Apoptosis was assessed by measurement of DNA fragmentation as described previously (Schmitz *et al.*, 2004). Briefly, apoptotic nuclei were prepared in a hypotonic lysis buffer (1% sodium citrate, 0.1% Triton X-100, 50 μ g/ml PI) and analyzed by flow cytometry. Nuclei to the left of the 2N peak, containing hypodiploid DNA, were considered apoptotic. PI uptake (2 μ g/ml) into nonfixed cells was evaluated by flow cytometric analyses with the FSC/FL2 profile (Wesselborg *et al.*, 1999). Flow cytometric analysis of cytochrome c release was carried out as published previously (Waterhouse and Trapani, 2003). For measurement of the mitochondrial membrane potential, cells were incubated with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; 5 μ g/ml; FL-1; Molecular Probes) for 20 min at room temperature in the dark, followed by analysis in a flow cytometer (FACScalibur, BD Biosciences). For flow cytometric analysis of Bak conformational change, cells were fixed in PBS/0.5% paraformaldehyde on ice for 30 min and subsequently washed three times in PBS/1% FCS. Staining with conformation-specific anti-Bak and isotype-matched control antibody was performed with a 1:50 dilution of the respective antibody in 50 μ l staining buffer (PBS, 1% FCS, 50 μ g/ml digitonin). Subsequently, cells were washed and incubated for 30 min in 50 μ l staining buffer containing 0.1 μ g Alexafluor 488-labeled chicken anti-mouse IgG.

Immunoblotting

Cells were lysed in lysis buffer (20 mM Tris/HCl, pH 7.4, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1 mM PMSF, and 1 μ g/ml each leupeptin, antipain, chymostatin, and pepstatin A) for 15 min on ice and centrifuged (15 min, 14,000 $\times$ g). For Western blot analysis postnuclear supernatants, equivalent to 1×10^6 cells or 30 μ g of protein, were loaded on a SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amersham Bioscience, Freiburg, Germany). The membrane was blocked with 5% BSA in Tris-buffered saline (TBS)/0.2% Tween for 2 h and incubated overnight with the primary antibodies at 4°C. Membranes were washed four times with TBS/0.02% Triton X-100 and incubated with the respective peroxidase-conjugated secondary antibody for 1 h. After extensive washing, the proteins were visualized by enhanced chemiluminescent staining using ECL reagents (Amersham Bioscience).

Caspase Activity Assay

Fluorogenic caspase assays were performed as described previously (Sohn *et al.*, 2005), using total cell extracts from cells treated with 2.5 μ M staurosporine or 100 μ M etoposide. Caspase-2-, -3-, and -9-like activities were measured using their respective substrates VDVAD-AMC, DEVD-AMC, and LEHD-AMC (Biomol, Hamburg, Germany). For the cleavage assays, 50 μ g of the cell extracts was dissolved in 200 μ l substrate buffer containing 50 mM HEPES, pH 7.3, 100 mM NaCl, 10% sucrose, 0.1% CHAPS, 10 mM DTT, and 50 μ M substrate. The reaction was incubated at 37°C and measured in triplicates in a Lambda Fluoro 320 Plus fluorimeter (Biotek, Bad Friedrichshall, Germany).

In Vivo Labeling of Active Caspases

To label the active site of caspases, 1×10^7 cells were incubated after apoptosis induction for an additional hour with 10 μ M biotin-VAD-fmk. Cells were harvested by centrifugation and extracted in 500 μ l lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM DTT) containing 2 μ g/ml the protease inhibitors aprotinin, leupeptin, pepstatin, and 1 mM phenylmethylsulfonyl fluoride. The biotinylated proteins were captured on 30 μ l streptavidin-conjugated agarose beads (Calbiochem, Bad Soden, Germany). After overnight rotation at 4°C the agarose beads were extensively washed in lysis buffer containing 0.5% NP-40. The biotinylated proteins were eluted from the beads by addition of 60 μ l SDS-sample buffer and incubation at 95°C for 10 min. Cell extracts, 25 μ g, or the eluted biotinylated proteins, 25 μ g, were used for SDS-PAGE and subsequent Western blot analysis.

Transmission Electron Microscopy

Transmission electron microscopy was performed as described previously (Schwerk and Schulze-Osthoff, 2005). Briefly, treated and untreated J16 and JMR cells were harvested, washed with PBS, and fixed in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C. Cells were further processed, embedded, and prepared using standard methods. Electron micrographs were taken using a Zeiss 902 electron microscope (Jena, Germany).

Figure 1. JMR cells are apoptosis-resistant to various inducers of genotoxic stress. (A) Induction of apoptosis: J16 and JMR cells were treated with 100 μ M etoposide, 1 μ M doxorubicin, 1 μ M daunorubicin, or 2.5 μ M staurosporine. After 24 h apoptosis was quantified by flow cytometric measurement of DNA fragmentation. The results show the means $\pm$ SD of triplicate samples and are representative of one of five independent experiments. (B) Measurement of DNA content: J16 and JMR cells were treated with 2.5 μ M staurosporine for 24 h or left untreated. Subsequently, cells were analyzed for the formation of hypodiploid DNA by PI staining. (C) Light microscopic pictures of J16 and JMR cells that were treated with etoposide or staurosporine for 48 and 24 h, respectively. (D) Detection of plasma membrane damage: J16 and JMR cells were treated with 100 μ M etoposide for 24 h or left untreated and then stained with PI. Flow cytometric analysis was carried out based on dye exclusion.

RESULTS

JMR Cells Are Resistant to Genotoxic Drugs

Jurkat cells are commonly used in signal transduction research of T-cells and were originally established from the peripheral blood of a 14-y-old boy with acute lymphoblastic leukemia (Schneider *et al.*, 1977). Jurkat cells are known to have a high degree of clonal heterogeneity (Parson *et al.*, 2005), and various subclones have been generated that are deficient in the expression of certain signaling molecules (Abraham and Weiss, 2004). We screened several clones of Jurkat cells obtained from ATCC (Manassas, VA) and other laboratories for their apoptosis sensitivity toward anticancer drugs. To this end, cells were treated for 20 h with 100 μ M etoposide, 1 μ M doxorubicin, 1 μ M daunorubicin, or 2.5 μ M staurosporine. Subsequent flow cytometric analyses identified a clone, designated JMR, which was completely resistant to apoptosis induction (Figure 1A). In contrast to the sensitive J16 Jurkat cells, JMR cells did not reveal apoptotic DNA fragmentation, but were resistant to all drugs tested (Figure 1A). However, JMR cells did sense noxious stimuli, as etoposide treatment resulted in accumulation of G2-arrested cells (Figure 1B).

In addition to DNA fragmentation, cells were analyzed by light microscopy for morphological alterations. J16 cells treated with etoposide or staurosporine showed typical apoptotic morphological changes, such as cell shrinkage, nuclear condensation, and membrane blebbing (Figure 1C). In contrast, JMR cells retained a normal morphology upon drug treatment, as did untreated cells, indicating that JMR

cells are resistant to genotoxic stress. To exclude necrosis as an alternative response, both cell lines were analyzed for membrane damage by the uptake of PI. In contrast to J16 cells, JMR cells excluded PI even after prolonged exposure to etoposide (Figure 1D).

JMR Cells Express Normal Levels of Bcl-2 and IAP Family Proteins, but Are Devoid of Caspase-9

Bcl-2 proteins are the major regulators of the mitochondrial apoptotic pathway, and their deregulated expression might result in altered sensitivity toward genotoxic drugs. Therefore, immunoblot analyses were carried out for the proapoptotic multidomain Bcl-2 family members Bak and Bax as well as for the anti-apoptotic proteins Bcl-2 and Bcl-x_L. As shown in Figure 2A, JMR cells and J16 cells expressed equivalent amounts of Bcl-2 and Bcl-x_L. Moreover, there were no significant differences in the expression levels of Bax and Bak between the two Jurkat cell clones. Another subgroup within the Bcl-2 family represents the proapoptotic BH3-only proteins, e.g., Bim and Bid. Western blot analyses showed no alterations in the expression of Bim and Bid in JMR cells, compared with J16 cells (Figure 2A). Thus, differences in the expression levels of Bcl-2 family proteins are presumably not responsible for the apoptosis-resistant phenotype of JMR cells.

We compared also the expression levels of inhibitor of apoptosis proteins (IAPs), which directly bind to and thereby inhibit active caspases (Deveraux and Reed, 1999). The expression of cIAP1, cIAP2, and XIAP did not differ

Figure 2. JMR cells are deficient of caspase-9. Total cell extracts of J16 and JMR cells were prepared and analyzed by Western blotting for (A) the Bcl-2 family members Bak, Bax, Bim, Bid, Bcl-2 and Bcl-x_L, for (B) the IAP family members cIAP1, cIAP2, and XIAP, and (C) for caspase-2, -3, -8, and -9 and Apaf-1. Equal protein loading of the blots was confirmed by staining for β -actin. (D) RNA was extracted from J16 and JMR cells and subjected to RT-PCR using oligonucleotide primers specific for caspase-9. The PCR products were separated on agarose gels and visualized by ethidium bromide staining. A GAPDH PCR product that was amplified in parallel served as a loading control.

between the two Jurkat clones (Figure 2B), indicating that the resistance of JMR cells was not due to up-regulation of IAPs. Another molecular hallmark of apoptosis is the activation of caspases. Caspase-2 and -3 as well caspase-8 were expressed at comparable levels in J16 and JMR cells. Striking, however, was the fact that expression of caspase-9, the essential initiator caspase in the mitochondrial pathway, was completely absent in JMR cells (Figure 2C). In contrast, Apaf-1, another essential component of the apoptosome, was equally expressed in J16 and JMR cells (Figure 2C). RT-PCR analysis showed that JMR cells were not only devoid of the caspase-9 protein, but also of its mRNA expression (Figure 2D). Therefore, resistance of JMR cells to apoptosis correlates with a genetic deficiency of caspase-9.

Staurosporine-mediated Caspase Activation Is Severely Impaired in JMR Cells

To investigate whether caspase-9 deficiency affects the activation of other caspases, J16 and JMR cells were treated with staurosporine for different time periods. Subsequently, immunoblot analyses were carried out for caspase-3, -8, and -9. A strong activation of all three caspases was observed in J16 cells within 2 h of staurosporine treatment, as shown by the appearance of the respective cleavage fragments of the individual caspases (Figure 3). In contrast, no proteolytic processing of caspase-3 or -8 was seen in caspase-9-deficient JMR cells (Figure 3). Caspase activation was also absent in JMR cells when measured by fluorescent substrate cleavage assays (see below). Taken together, these results suggest that Jurkat cells depend on caspase-9 for the execution of apoptosis induced by stimulation of the intrinsic death pathway.

Although staurosporine-treated JMR cells appeared morphologically normal as assessed by light microscopy (Figure 1C) and did not show any signs of necrosis, we wanted to investigate whether the cellular ultrastructure was affected by drug treatment. To this end, J16 and JMR cells were treated overnight with staurosporine and subjected to transmission electron microscopy. J16 cells were completely dismantled and showed highly condensed chromatin (Figure 4). JMR cells, in contrast, appeared normal except that staurosporine-treated cells contained slightly enlarged nuclei

Figure 3. Caspase activation is severely impaired in JMR cells. J16 and JMR cells were stimulated with 2.5 μ M staurosporine for the indicated times. Total cell extracts were separated by SDS-PAGE and blotted for caspase-3 (top panel), -8 (middle panel), and -9 (bottom panel). Closed and open arrowheads, the zymogens and the cleaved forms of the individual caspases, respectively. Blots were probed for β -actin as a loading control.

rosaporine-treated cells contained slightly enlarged nuclei (Figure 4). Importantly, no vacuoles were observed suggesting that caspase-9-deficient JMR cells do not switch to autophagy upon staurosporine treatment.

Upstream Mitochondrial Apoptotic Events Are Not Altered in JMR Cells

Because activation of the caspase cascade was impaired in JMR cells, we investigated whether events further upstream

Figure 4. Preserved ultrastructure of JMR cells after drug treatment. J16 and JMR cells were treated for 24 h with 2.5 μ M staurosporine or left untreated. Subsequently, cells were analyzed by electron microscopy. Note the pronounced chromatin condensation in staurosporine-stimulated J16 cells that is completely absent in JMR cells.

Figure 5. Mitochondrial membrane permeabilization, but not Bak activation or cytochrome c release requires caspase-9. (A) Measurement of Bak activation: J16 and JMR cells were treated for 2 h with 2.5 μM staurosporine (open histograms) or left untreated (filled histograms). Subsequently, cells were permeabilized with digitonin, incubated with a conformation-specific Bak antibody, and analyzed by flow cytometry. No staining was detected with an isotype-matched control antibody. (B) Detection of cytochrome c release: Cells were stimulated as described in A and analyzed for cytochrome c release in a flow cytometer. Filled histograms, the staining of the untreated cells; open histograms, the results of the staurosporine-treated cells. (C) Measurement of DNA fragmentation: J16 and JMR cells were treated in parallel with 2.5 μM staurosporine for 24 h and monitored for the formation of hypodiploid DNA. (D) Measurement of $\Delta\Psi_M$ loss: Cells were treated as in A, stained with JC1 and analyzed for the loss of the $\Delta\Psi_M$ by flow cytometry. (E) J16 and JMR cells were treated for 20 h with 2.5 μM staurosporine and $\Delta\Psi_M$ was measured as in D.

in the mitochondrial pathway were also affected by caspase-9 deficiency. Mitochondrial apoptosis is regulated by the multidomain Bcl-2 proteins Bax or Bak, which undergo a N-terminal conformational change, resulting in their homo-oligomerization and the subsequent release of proapoptotic proteins from the mitochondrial intermembrane space (Cory *et al.*, 2003). The conformational change and activation of Bak can be analyzed with specific antibodies against their normally occluded N-terminus. Flow cytometric analysis with such antibodies revealed that upon staurosporine treatment Bak was activated not only in J16 cells, but also in JMR cells (Figure 5A). Next, we analyzed whether activation of Bak leads to permeabilization of mitochondria and cytochrome c release. As assessed by flow cytometry, J16 cells as well as JMR cells released cytochrome c within 2 h upon treatment with staurosporine (Figure 5B). Therefore, apoptotic events upstream of the apoptosome proceed normally in JMR cells. To validate that JMR cells were resistant to staurosporine treatment in this experiment, cells were incubated for an additional 6 h to monitor DNA fragmen-

tation. Indeed, despite the fact that mitochondria were permeabilized, JMR cells did not exhibit significant DNA fragmentation (Figure 5C).

$\Delta\Psi_M$ of JMR Cells Is Not Altered during Genotoxic Stress

In addition to the release of proapoptotic proteins from the intermembrane space, the loss of $\Delta\Psi_M$ is a typical and early event during apoptosis (Zamzami *et al.*, 1995, 1996). Disruption of $\Delta\Psi_M$ suggests that the integrity of the inner mitochondrial membrane is affected. Therefore, we measured $\Delta\Psi_M$ as an additional parameter for mitochondrial integrity. To this end, J16 and JMR cells were stimulated with staurosporine for 2 h and then stained with the $\Delta\Psi_M$ -sensitive dye JC-1. As assessed by flow cytometry, a rapid loss of $\Delta\Psi_M$ could be observed in J16 cells upon stimulation with staurosporine. In contrast, $\Delta\Psi_M$ did not decrease in JMR cells (Figure 5D). Even after prolonged treatment with staurosporine or etoposide, the $\Delta\Psi_M$ remained intact in JMR cells (Figure 5E). Thus, caspase-9 deficiency seems to uncouple

Figure 6. Reexpression of caspase-9 in JMR cells restores apoptosis and $\Delta\Psi_M$ loss. (A) JMR cells and caspase-9–reconstituted JMR/C9 cells were treated with 2.5 μ M staurosporine or 100 μ M etoposide for 24 and 48 h, respectively. Light microscopic pictures were taken at a 200-fold magnification. (B) Caspase-9–reconstituted JMR/C9 cells were treated with 2.5 μ M staurosporine for 2 h (open histograms) or left untreated (closed histograms) and analyzed for Bak activation (top left panel), cytochrome c release (top right panel), mitochondrial membrane potential (bottom left panel) and PI exclusion (bottom right panel). The analyses were performed by flow cytometry as described in Figure 4. (C) J16, JMR, and JMR/C9 cells were treated with 2.5 μ M staurosporine or 100 μ M etoposide for the indicated time points. Apoptosis was assessed by measurement of DNA fragmentation and flow cytometry. The results show the means $\pm$ SEM of two independent experiments. (D) J16, JMR, and JMR/C9 cells were treated for 16 h with 2 ng/ml CD95L (■) or left untreated (□). Apoptosis was measured as in C.

cytochrome c release and loss of $\Delta\Psi_M$ as two separate events in apoptosis.

Stable Transfection of Caspase-9 Restores Sensitivity for Genotoxic Stress in JMR Cells

To verify that the resistance to anticancer drugs in JMR cells was due to the absence of caspase-9, JMR cells were stably transfected with a caspase-9 expression construct. When treated with etoposide or staurosporine, caspase-9–reconstituted JMR/C9 cells showed cell shrinkage, nuclear condensation, membrane blebbing, and eventual demise comparable to J16 cells (Figure 6A). On incubation with staurosporine for 2 h, caspase-9–reconstituted JMR cells displayed Bak activation, cytochrome c release and, importantly, also showed a rapid loss of $\Delta\Psi_M$ (Figure 6B), similar to J16 cells (see Figure 5). After 8 h of treatment the majority of caspase-9–retransfected cells became PI-positive and underwent cell death (Figure 6B). Flow cytometric analyses of DNA fragmentation also indicated that apoptosis proceeded with comparable kinetics in J16 and JMR/C9 cells, while the parental JMR cells showed no signs of DNA fragmentation upon treatment with staurosporine or etoposide (Figure 6C).

In addition, we investigated whether the absence of caspase-9 had any influence on death receptor-mediated apoptosis. To this end, we treated J16, JMR, and JMR/C9 cells with CD95L. Interestingly, CD95L-induced apoptosis was severely impaired in JMR cells (Figure 6D), which is in consistent with the fact that Jurkat cells are type II cells (Scaffidi *et al.*, 1998).

To analyze caspase activation in J16, JMR, and JMR/C9 cells, fluorescent substrate cleavage assays were performed with extracts from cells treated with either staurosporine or etoposide for different periods of time. Caspase-3-like (DEVDase) activity was observed in lysates from J16 and JMR/C9 cells after etoposide as well as after staurosporine treatment (Figure 7A). This was also true for caspase-9-like (LEHDase) activities. Even after prolonged stimulation with staurosporine or etoposide, JMR cells showed caspase activities for both substrates that were even lower than the background levels in unstimulated J16 and JMR/C9 cells (Figure 7A). Activation of caspases in response to etoposide or staurosporine treatment was also tested by immunoblotting. Western blot analysis showed proteolytic activation of

caspase-9 in JMR/C9 cells upon etoposide treatment. The cleaved forms, p37 and p35, appeared with kinetics similar to those of J16 cells (Figure 7B). In addition, caspase-3 activation, as shown by the appearance of its processed p19 and p17 fragments, was similar in J16 and JMR/C9 cells, but absent in JMR cells (Figure 7B). Activation of caspase-9 and -3 was observed also in JMR/C9 cells, but not in parental JMR cells when treated with staurosporine (Figure 7C). Thus, the presence of caspase-9 is essential for activation of downstream effector caspases such as caspase-3.

Activation of Caspase-2 Is Dependent on Downstream Caspase Activation

Recently, an initiator role has also been suggested for caspase-2 in stress-induced apoptosis (Lassus *et al.*, 2002; Robertson *et al.*, 2002), whereas other reports proposed that caspase-2 acts downstream of mitochondria. Because caspase-9–deficient cells represent an ideal system to analyze whether caspase-2 activation occurs upstream or downstream of apoptosome function, we treated J16, JMR, and JMR/C9 cells with etoposide and analyzed caspase-2 processing. Pro-caspase-2 was processed into the p32 intermediate and the p19 active fragments in J16 and JMR/C9 cells, but not in the parental JMR cell line (Figure 8A). Additionally, analysis of caspase-2 cleavage activity using the fluorometric substrate VDVAD-AMC, confirmed the lack of caspase-2 activation in JMR cells, but revealed significant caspase-2 activity in J16 and JMR/C9 cells during etoposide-induced apoptosis (Figure 8B).

Recent investigations suggested that dimerization, but not proteolytic cleavage is an initial event for the activation of caspase-2 and other initiator caspases (Baliga *et al.*, 2004). To examine whether caspase-2 was activated in the absence of proteolytic cleavage, we used an *in vivo* affinity labeling approach, using the biotinylated caspase inhibitor biotin-VAD-fmk that detects only active caspases. J16, JMR, and JMR/C9 cells were treated with etoposide for 6 h and incubated the cells with biotin-VAD-fmk for an additional hour. The affinity-labeled caspases were then precipitated with streptavidin agarose beads and immunoblotted for caspase-2 (Figure 8C). A moderate labeling of full-length caspase-2, which contains a low proteolytic activity compared with the processed caspase-2 (Baliga *et al.*, 2004), was detected in un-

Figure 7. Reconstitution of caspase-9 restores caspase processing and activation in JMR cells. (A) J16 (□), JMR cells (■), and caspase-9-reconstituted JMR/C9 cells (▨) were treated for the indicated time with 100 μ M etoposide (top panels) or 2.5 μ M staurosporine (bottom panels). Cellular extracts were then subjected to fluorescence-based enzyme assays for caspase-3-like activities with the substrate DEVD-amc, and for caspase-9-like activities with the substrate LEHD-amc. (B) JMR and JMR/C9 cells were treated with 100 μ M etoposide for the indicated time points. Total cell extracts were separated by SDS-PAGE and immunoblotted for caspase-3 (top panel) and -9 (middle panel). (C) JMR and JMR/C9 cells were treated with 2.5 μ M staurosporine for the indicated times and blotted for caspase-3 (top panel) and -9 (middle panel). The asterisk denotes a nonspecific protein that was detected by the antibody. Blots were probed for β -actin as a loading control (bottom panels in B and C). Closed and open arrowheads, the zymogen forms and cleaved forms of the indicated caspases, respectively.

stimulated J16, JMR/C9, and JMR cells and might be explained by spontaneous dimerization of caspase-2 during the experimental procedure (Tinel and Tschopp, 2004). Biotin-VAD-fmk-labeled unprocessed procaspase-2 was observed in etoposide-treated JMR cells as well. However, the amount of biotin-VAD-fmk-labeled caspase-2 was similar in untreated and etoposide-treated JMR cells, suggesting that this caspase-2 activation was not related to genotoxic stress. Importantly, biotin-VAD-fmk-labeled and hence fully active caspase-2 fragments were detected in etoposide-treated J16 and JMR/C9 cells, but not in untreated cells, as shown by appearance of the precipitated p19 fragment (Figure 8C). No

labeling of active caspase-2 cleavage fragments was observed in caspase-9-deficient JMR cells, indicating that caspase-2 was not activated in the cells (Figure 8C). In agreement with our previous findings, active caspase-3 fragments (p21/p19/p17/p12) were detected in precipitates from etoposide-treated J16 and JMR/C9 cells, but not in the parental JMR cells. Next, we asked whether caspase-2 could be activated in JMR cells by other inducers of cytotoxic stress. To this end, J16 and JMR cells were treated with doxorubicin and staurosporine (Figure 8D). The active p19 fragment of caspase-2 was observed in caspase-9-proficient J16 cells but not in caspase-9-deficient JMR cells upon doxorubicin and

Figure 8. Genotoxic stress-induced caspase-2 processing and activation is dependent on caspase-9. (A) Proteolytic processing of caspase-2: J16, JMR, and JMR/C9 cells were treated with 100 μ M etoposide for the indicated time, before cells extracts were prepared and immunoblotted for caspase-2. Arrowheads indicate the proform and the p32 and p19 proteolytic fragments of the caspase-2. β -actin served as a loading control. (B) Detection of caspase-2-like activity. Cells were treated with etoposide for the indicated time, and caspase-2 activity was measured in a fluorimeter by incubating the cell extracts with the substrate VDVAD-amc. The results show the means $\pm$ SD of triplicate samples and are representative of one of more than two independent experiments. (C) In vivo peptide affinity labeling of active caspases: Each, 1×10^7 J16, JMR, and JMR/C9 cells were left untreated (C) or incubated with 100 μ M etoposide (Etop) for 6 h. After the incubation, active caspases were labeled in vivo by the addition of biotin-VAD-fmk for 1 h. Cell extracts were prepared and the bio-

tylated proteins were captured with streptavidin agarose beads. The streptavidin beads (S) and 25 μ g protein from the input material (I) were analyzed by SDS-PAGE and assayed for the activation of caspase-2 and -3 by Western blotting. Closed and open arrowheads, the zymogens and active forms of the caspases, respectively. The asterisk denotes a nonspecific protein that was detected by the caspase-2 antibody. (D) J16 and JMR cells were treated for 8 h with 1 μ M doxorubicin or 1 μ M staurosporine or were left untreated. Cellular lysates were analyzed by Western blot with a caspase-2-specific antibody. Anti-tubulin was used as a loading control.

staurosporine treatment. These results therefore clearly demonstrate that caspase-2 is not activated in the absence of caspase-9, but rather requires the apoptosome pathway for genotoxic stress-induced apoptosis.

DISCUSSION

In this study, we identified a Jurkat T-cell line deficient in caspase-9 expression that is, to our knowledge, the first caspase-9-lacking tumor cell line and thus represents an excellent functional complementation to the previously described caspase-8-deficient Jurkat cells (Juo *et al.*, 1998). Short tandem repeat DNA profiling verified that the caspase-9-deficient JMR cells were derived from Jurkat cells (data not shown). Using JMR cells we show that caspase-9 is indispensable for genotoxic stress-induced apoptosis. This is consistent with the initial characterization of fibroblasts or embryonic stem cells from caspase-9 and Apaf-1 null mice that are resistant to a broad range of cytotoxic agents (Cecconi *et al.*, 1998; Hakem *et al.*, 1998; Kuida *et al.*, 1998; Yoshida *et al.*, 1998; Soengas *et al.*, 1999). Nevertheless, subsequent studies hinted at the possibility that additional, context-specific pathways eventually exist that could operate in the absence of either caspase-9 or Apaf-1. Caspase-9 activation was found to be uncoupled from Apaf-1 in Sendai-virus induced apoptosis (Bitzer *et al.*, 2002). Furthermore, Apaf-1-deficient fibroblasts but not myoblasts were protected from apoptosis induced by cytotoxic drugs or E2F1 overexpression (Ho *et al.*, 2004). Marsden *et al.* (2002) detected marginal caspase-7 activation in caspase-9- and Apaf-1-deficient thymocytes and speculated that caspase-7 activation might be due to an alternative intrinsic pathway that bypasses the apoptosome. Although different Apaf-1-related molecules exist, the identity of such alternative pathways remains unknown. The caspase-9-deficient Jurkat line JMR will certainly prove to be useful for further analysis of apoptosis signaling pathways in the absence of apoptosome function.

We used JMR cells to address two highly contentious issues of apoptosis regulation. First, we show that, unlike cytochrome c release, loss of $\Delta\Psi_M$ is dependent on caspase activation *in vivo*. The loss of caspase-9 allowed us to clearly separate cytochrome c release from the decrease of $\Delta\Psi_M$, suggesting that loss of $\Delta\Psi_M$ and mitochondrial outer membrane permeabilization (MOMP) are two independent and consecutive events. Although it is well established that anti-apoptotic Bcl-2 members control MOMP, there is a major controversy between the interrelationship of MOMP and loss of $\Delta\Psi_M$. Several reports suggested that both events are coupled and that even cytochrome c release is dependent on the loss of $\Delta\Psi_M$ (Zamzami *et al.*, 1995; Zamzami and Kroemer, 2001). Also the role of caspases in both processes has been unclear. It was reported that both, cytochrome c release and mitochondrial depolarization, depend on caspase activation (Marsden *et al.*, 2002, 2004), whereas cytochrome c release was also demonstrated to occur earlier than caspase activation and mitochondrial membrane depolarization (Bossy-Wetzel *et al.*, 1998). Interestingly, blocking caspase activity by zVAD-fmk prevented loss of $\Delta\Psi_M$, but not cytochrome c release. In fact it was shown that this disruption of mitochondrial function might be caused by the caspase-3-mediated cleavage of a 75-kDa subunit of complex I in the electron transport chain (Ricci *et al.*, 2003, 2004).

The opposing data have led to two models of mitochondrial permeabilization during apoptosis. In one model, MOMP has been suggested to occur by the opening of the permeability transition pore (PTP), a channel spanning the outer and the inner mitochondrial membrane. This PTP

model suggests that the release of cytochrome c and the loss of $\Delta\Psi_M$ are coupled events (Zamzami and Kroemer, 2001). The other model implies that only the outer mitochondrial membrane is permeabilized by proapoptotic Bcl-2 proteins, whereas mitochondrial membrane depolarization is a secondary event. It should be noted that most of these studies were done in cell-free systems or in permeabilized cells. Our data strongly argue against the PTP model and support a model in which the permeabilization of the outer membrane is sufficient for cytochrome c release without the requirement of additional events, including the loss of $\Delta\Psi_M$. Moreover, because cytochrome c release occurred with similar kinetics in JMR and J16 cells, our data also argue against a caspase-dependent amplification loop of cytochrome c release. In agreement with our results, it was shown that in granzyme B-induced apoptosis a transient loss of $\Delta\Psi_M$ could be regenerated by zVAD-fmk, despite the fact that cytochrome c had been released into the cytosol (Waterhouse *et al.*, 2006).

We further used caspase-9-deficient JMR cells to investigate the role of caspase-2 in genotoxic stress-induced apoptosis. Some reports had suggested that caspase-2 plays a crucial role in DNA-damage-induced apoptosis (Lassus *et al.*, 2002; Robertson *et al.*, 2000). Moreover, a recent study showed that caspase-2 is recruited to and activated at the CD95 DISC (Lavrik *et al.*, 2006). Supportive for an initiator function is the sequence of caspase-2, which contains a CARD motif that might assist dimerization of caspase-2 upon interaction with different adapter proteins including RAIDD or others (Read *et al.*, 2002; Baliga *et al.*, 2004; Zhivotovsky and Orrenius, 2005). Caspase-2 has also been suggested to mediate the function of p53, which transcriptionally activates the death domain protein PIDD (Tinel and Tschopp, 2004). Up-regulated PIDD, together with RAIDD or RIP1, can form a multiprotein complex, called the PIDDosome, which activates either caspase-2 or transcription factor NF- κ B (Tinel and Tschopp, 2004; Janssens *et al.*, 2005). Biochemical studies using RNAi or antisense strategies placed caspase-2 upstream of mitochondria and cytochrome c release (Lassus *et al.*, 2002; Robertson *et al.*, 2002; Lin *et al.*, 2004). In line with this, it was shown that recombinant caspase-2 was able to release cytochrome c in a Bcl-2-independent manner in permeabilized cells (Enoksson *et al.*, 2004; Robertson *et al.*, 2004). However, it cannot be excluded that recombinant caspase-2 activates other caspases in permeabilized cells or, by its ability to activate Bid, mediates cytochrome c release. Surprisingly, although one study showed that chemically inactivated caspase-2 still released cytochrome c (Robertson *et al.*, 2004), this was not observed with genetically inactivated caspase-2 (Enoksson *et al.*, 2004). Therefore, it remains unclear whether caspase-2 is directly involved in cytochrome c release. It is noteworthy that most studies suggesting a requirement of caspase-2 in stress-induced apoptosis used a single and identical small-interfering (si) RNA sequence (Lassus *et al.*, 2002; Lin *et al.*, 2004, 2005). A recent correction to one of these studies, however, pointed out that other siRNA sequences that reduced caspase-2 levels in a similar manner in the same cell type failed to influence cell death induced by genotoxic stress (Lassus *et al.*, 2004).

Unlike caspase-8 or -9, caspase-2 obviously does not directly cleave another mammalian caspase, aside from its own precursor (Ricci *et al.*, 2003). However, procaspase-2 is efficiently cleaved by caspase-3 *in vitro* (Slee *et al.*, 1999), and in many experimental systems caspase-2 cleavage is detected downstream of caspase-3. For instance, it was shown that caspase-2 processing depends on caspase-3 and -9 during UV- and TNF- α -induced apoptosis (Paroni *et al.*, 2001). Moreover, expression of dominant-negative caspase-9 inhib-

ited caspase-2 activation upon stimuli of the intrinsic, but not the extrinsic pathway (Werner *et al.*, 2004). In line with this, no caspase-2 processing has been observed in irradiated thymocytes from Apaf-1 or caspase-9 knockout mice (O'Reilly *et al.*, 2002) or from Bax/Bak double-deficient fibroblasts (Ruiz-Vela *et al.*, 2005). Although most of these previous studies investigated caspase-2 processing as a measure of its activation, our experiments using peptide affinity labeling clearly suggest that caspase-2 also fails to acquire catalytic activity in the absence of caspase-9. Interestingly, a recent study (Tu *et al.*, 2006) failed to observe activation of caspase-2 in several settings where it had been implicated, including genotoxic stress. In contrast, caspase-2 was identified as a specific initiator caspase for heat-shock-induced apoptosis, in which it mediated MOMP and caspase-3 activation in a strictly Bid-dependent manner (Bonzon *et al.*, 2006; Tu *et al.*, 2006).

Thus, our data demonstrate that caspase-2 cannot bypass the apoptosome, but depends on caspase-9. Certainly, we cannot exclude the possibility that the role of caspase-2 in genotoxic stress-induced apoptosis might be cell type- or stimulus-specific; however, studies proposing an initiator role of caspase-2 were also performed in Jurkat cells using the same apoptotic stimuli as in our study (Robertson *et al.*, 2002; Lin *et al.*, 2004; Tinel and Tschopp, 2004). It might be argued that a minor amount of caspase-2 activation is sufficient to trigger the caspase cascade. We consider this possibility unlikely, because overexpression of caspase-2 is generally required to induce cell death (Baliga *et al.*, 2004). Recently, it was reported that caspase-2 is an activator of NF- κ B and p38 kinase (Lamkanfi *et al.*, 2005), suggesting that caspase-2 might act as a proinflammatory rather than as an apoptotic caspase. This assumption would not only be consistent with the lack of an apoptotic phenotype in caspase-2 null mice (Bergeron *et al.*, 1998), but also with the sequence of caspase-2 that is more closely related to the proinflammatory than to the proapoptotic initiator caspases (Lamkanfi *et al.*, 2002).

ACKNOWLEDGMENTS

We are grateful to Daniel Scholtysik and Carina Meyer for their expert technical assistance. We thank Dr. Guy Salvesen for the caspase-9 plasmid, Dr. Harald Wajant for CD95L, and Dr. Wilhelm G. Dirks for DNA profiling of JMR cells. This study was supported by grants from the Deutsche Krebshilfe, the Deutsche Forschungsgemeinschaft (SFB503, SFB575), and the Forschungsskommission of the Medical School Düsseldorf.

REFERENCES

- Abraham, R. T., and Weiss, A. (2004). Jurkat T cells and development of the T-cell receptor signalling paradigm. *Nat. Rev. Immunol.* 4, 301–308.
- Baliga, B. C., Read, S. H., and Kumar, S. (2004). The biochemical mechanism of caspase-2 activation. *Cell Death Differ.* 11, 1234–1241.
- Bergeron, L., *et al.* (1998). Defects in regulation of apoptosis in caspase-2-deficient mice. *Genes Dev.* 12, 1304–1314.
- Bitzer, M., *et al.* (2002). Caspase-8 and Apaf-1-independent caspase-9 activation in Sendai virus-infected cells. *J. Biol. Chem.* 277, 29817–29824.
- Boatright, K. M., *et al.* (2003). A unified model for apical caspase activation. *Mol. Cell* 11, 529–541.
- Bonzon, C., Bouchier-Hayes, L., Pagliari, L. J., Green, D. R., and Newmeyer, D. D. (2006). Caspase-2-induced apoptosis requires Bid cleavage: a physiological role for Bid in heat shock-induced death. *Mol. Biol. Cell* 17, 2150–2157.
- Bossy-Wetzel, E., Newmeyer, D. D., and Green, D. R. (1998). Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J.* 17, 37–49.
- Cecconi, F., Alvarez-Bolado, G., Meyer, B. I., Roth, K. A., and Gruss, P. (1998). Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. *Cell* 94, 727–737.
- Cory, S., Huang, D. C., and Adams, J. M. (2003). The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene* 22, 8590–8607.
- Deveraux, Q. L., and Reed, J. C. (1999). IAP family proteins—suppressors of apoptosis. *Genes Dev.* 13, 239–252.
- Donepudi, M., Mac Sweeney, A., Briand, C., and Grutter, M. G. (2003). Insights into the regulatory mechanism for caspase-8 activation. *Mol. Cell* 11, 543–549.
- Enoksson, M., Robertson, J. D., Gogvadze, V., Bu, P., Kropotov, A., Zhivotovsky, B., and Orrenius, S. (2004). Caspase-2 permeabilizes the outer mitochondrial membrane and disrupts the binding of cytochrome c to anionic phospholipids. *J. Biol. Chem.* 279, 49575–49578.
- Fischer, U., Janicke, R. U., and Schulze-Osthoff, K. (2003). Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ.* 10, 76–100.
- Fuentes-Prior, P., and Salvesen, G. S. (2004). The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochem. J.* 384, 201–232.
- Guo, Y., Srinivasula, S. M., Druihe, A., Fernandes-Alnemri, T., and Alnemri, E. S. (2002). Caspase-2 induces apoptosis by releasing proapoptotic proteins from mitochondria. *J. Biol. Chem.* 277, 13430–13437.
- Hakem, R., *et al.* (1998). Differential requirement for caspase 9 in apoptotic pathways in vivo. *Cell* 94, 339–352.
- Ho, A. T., Li, Q. H., Hakem, R., Mak, T. W., and Zacksenhaus, E. (2004). Coupling of caspase-9 to Apaf1 in response to loss of pRb or cytotoxic drugs is cell-type-specific. *EMBO J.* 23, 460–472.
- Janssens, S., Tinel, A., Lippens, S., and Tschopp, J. (2005). PIDD mediates NF-kappaB activation in response to DNA damage. *Cell* 123, 1079–1092.
- Juo, P., Kuo, C. J., Yuan, J., and Blenis, J. (1998). Essential requirement for caspase-8/FLICE in the initiation of the Fas-induced apoptotic cascade. *Curr. Biol.* 8, 1001–1008.
- Kuida, K., Haydar, T. F., Kuan, C. Y., Gu, Y., Taya, C., Karasuyama, H., Su, M. S., Rakic, P., and Flavell, R. A. (1998). Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* 94, 325–337.
- Lamkanfi, M., D'Hondt, K., Vande Walle, L., van Gurp, M., Denecker, G., Demeulemeester, J., Kalai, M., Declercq, W., Saelens, X., and Vandennebe, P. (2005). A novel caspase-2 complex containing TRAF2 and RIP1. *J. Biol. Chem.* 280, 6923–6932.
- Lamkanfi, M., Declercq, W., Kalai, M., Saelens, X., and Vandennebe, P. (2002). Alice in caspase land. A phylogenetic analysis of caspases from worm to man. *Cell Death Differ.* 9, 358–361.
- Lassus, P., Opitz-Araya, X., and Lazebnik, Y. (2002). Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* 297, 1352–1354.
- Lassus, P., Opitz-Araya, X., and Lazebnik, Y. (2004). Corrections and clarifications. *Science* 306, 1683.
- Lavrik, I. N., Golks, A., Baumann, S., and Krammer, P. H. (2006). Caspase-2 is activated at the CD95 death-inducing signaling complex in the course of CD95-induced apoptosis. *Blood* 108, 559–565.
- Leist, M., and Jaattela, M. (2001). Four deaths and a funeral: from caspases to alternative mechanisms. *Nat. Rev. Mol. Cell Biol.* 2, 589–598.
- Lin, C. F., Chen, C. L., Chang, W. T., Jan, M. S., Hsu, L. J., Wu, R. H., Fang, Y. T., Tang, M. J., Chang, W. C., and Lin, Y. S. (2005). Bcl-2 rescues ceramide- and etoposide-induced mitochondrial apoptosis through blockage of caspase-2 activation. *J. Biol. Chem.* 280, 23758–23765.
- Lin, C. F., Chen, C. L., Chang, W. T., Jan, M. S., Hsu, L. J., Wu, R. H., Tang, M. J., Chang, W. C., and Lin, Y. S. (2004). Sequential caspase-2 and caspase-8 activation upstream of mitochondria during ceramide and etoposide-induced apoptosis. *J. Biol. Chem.* 279, 40755–40761.
- Marsden, V. S., Ekert, P. G., Van Delft, M., Vaux, D. L., Adams, J. M., and Strasser, A. (2004). Bcl-2-regulated apoptosis and cytochrome c release can occur independently of both caspase-2 and caspase-9. *J. Cell Biol.* 165, 775–780.
- Marsden, V. S., *et al.* (2002). Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome. *Nature* 419, 634–637.
- Martinou, J. C., and Green, D. R. (2001). Breaking the mitochondrial barrier. *Nat. Rev. Mol. Cell Biol.* 2, 63–67.
- Newmeyer, D. D., and Ferguson-Miller, S. (2003). Mitochondria: releasing power for life and unleashing the machineries of death. *Cell* 112, 481–490.

- O'Reilly, L. A., *et al.* (2002). Caspase-2 is not required for thymocyte or neuronal apoptosis even though cleavage of caspase-2 is dependent on both Apaf-1 and caspase-9. *Cell Death Differ.* 9, 832–841.
- Paroni, G., Henderson, C., Schneider, C., and Brancolini, C. (2001). Caspase-2-induced apoptosis is dependent on caspase-9, but its processing during UV- or tumor necrosis factor-dependent cell death requires caspase-3. *J. Biol. Chem.* 276, 21907–21915.
- Parson, W., Kirchebner, R., Muhlmann, R., Renner, K., Kofler, A., Schmidt, S., and Kofler, R. (2005). Cancer cell line identification by short tandem repeat profiling: power and limitations. *FASEB J.* 19, 434–436.
- Peter, M. E., and Krammer, P. H. (2003). The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ.* 10, 26–35.
- Read, S. H., Baliga, B. C., Ekert, P. G., Vaux, D. L., and Kumar, S. (2002). A novel Apaf-1-independent putative caspase-2 activation complex. *J. Cell Biol.* 159, 739–745.
- Ricci, J. E., Gottlieb, R. A., and Green, D. R. (2003). Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis. *J. Cell Biol.* 160, 65–75.
- Ricci, J. E., Munoz-Pinedo, C., Fitzgerald, P., Bailly-Maitre, B., Perkins, G. A., Yadava, N., Scheffler, I. E., Ellisman, M. H., and Green, D. R. (2004). Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain. *Cell* 117, 773–786.
- Robertson, J. D., Enoksson, M., Suomela, M., Zhivotovsky, B., and Orrenius, S. (2002). Caspase-2 acts upstream of mitochondria to promote cytochrome c release during etoposide-induced apoptosis. *J. Biol. Chem.* 277, 29803–29809.
- Robertson, J. D., Gogvadze, V., Kropotov, A., Vakifahmetoglu, H., Zhivotovsky, B., and Orrenius, S. (2004). Processed caspase-2 can induce mitochondria-mediated apoptosis independently of its enzymatic activity. *EMBO Rep.* 5, 643–648.
- Robertson, J. D., Gogvadze, V., Zhivotovsky, B., and Orrenius, S. (2000). Distinct pathways for stimulation of cytochrome c release by etoposide. *J. Biol. Chem.* 275, 32438–32443.
- Ruiz-Vela, A., Opferman, J. T., Cheng, E. H., and Korsmeyer, S. J. (2005). Proapoptotic BAX and BAK control multiple initiator caspases. *EMBO Rep.* 6, 379–385.
- Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Krammer, P. H., and Peter, M. E. (1998). Two CD95 (APO-1/Fas) signaling pathways. *EMBO J.* 17, 1675–1687.
- Schmitz, I., Weyd, H., Krueger, A., Baumann, S., Fas, S. C., Krammer, P. H., and Kirchhoff, S. (2004). Resistance of short term activated T cells to CD95-mediated apoptosis correlates with de novo protein synthesis of c-FLIPshort. *J. Immunol.* 172, 2194–2200.
- Schneider, U., Schwenk, H. U., and Bornkamm, G. (1977). Characterization of EBV-genome negative “null” and “T” cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. *Int. J. Cancer* 19, 621–626.
- Schwerk, C., and Schulze-Osthoff, K. (2005). Methyltransferase inhibition induces p53-dependent apoptosis and a novel form of cell death. *Oncogene* 24, 7002–7011.
- Slee, E. A., *et al.* (1999). Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J. Cell Biol.* 144, 281–292.
- Soengas, M. S., Alarcon, R. M., Yoshida, H., Giaccia, A. J., Hakem, R., Mak, T. W., and Lowe, S. W. (1999). Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. *Science* 284, 156–159.
- Sohn, D., Schulze-Osthoff, K., and Janicke, R. U. (2005). Caspase-8 can be activated by interchain proteolysis without receptor-triggered dimerization during drug-induced apoptosis. *J. Biol. Chem.* 280, 5267–5273.
- Thornberry, N. A., *et al.* (1997). A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J. Biol. Chem.* 272, 17907–17911.
- Tinel, A., and Tschoopp, J. (2004). The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress. *Science* 304, 843–846.
- Tu, S., McStay, G. P., Boucher, L. M., Mak, T., Beere, H. M., and Green, D. R. (2006). In situ trapping of activated initiator caspases reveals a role for caspase-2 in heat shock-induced apoptosis. *Nat. Cell Biol.* 8, 72–77.
- Wagner, K. W., Engels, I. H., and Deveraux, Q. L. (2004). Caspase-2 can function upstream of bid cleavage in the TRAIL apoptosis pathway. *J. Biol. Chem.* 279, 35047–35052.
- Wang, X. (2001). The expanding role of mitochondria in apoptosis. *Genes Dev.* 15, 2922–2933.
- Waterhouse, N. J., Sedelies, K. A., Sutton, V. R., Pinkoski, M. J., Thia, K. Y., Johnstone, R., Bird, P. I., Green, D. R., and Trapani, J. A. (2006). Functional dissociation of DeltaPsim and cytochrome c release defines the contribution of mitochondria upstream of caspase activation during granzyme B-induced apoptosis. *Cell Death Differ.* 13, 607–618.
- Waterhouse, N. J., and Trapani, J. A. (2003). A new quantitative assay for cytochrome c release in apoptotic cells. *Cell Death Differ.* 10, 853–855.
- Werner, A. B., Tait, S. W., de Vries, E., Eldering, E., and Borst, J. (2004). Requirement for aspartate-cleaved bid in apoptosis signaling by DNA-damaging anti-cancer regimens. *J. Biol. Chem.* 279, 28771–28780.
- Wesselborg, S., Engels, I. H., Rossmann, E., Los, M., and Schulze-Osthoff, K. (1999). Anticancer drugs induce caspase-8/FLICE activation and apoptosis in the absence of CD95 receptor/ligand interaction. *Blood* 93, 3053–3063.
- Yoshida, H., Kong, Y. Y., Yoshida, R., Elia, A. J., Hakem, A., Hakem, R., Penninger, J. M., and Mak, T. W. (1998). Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell* 94, 739–750.
- Zamzami, N., and Kroemer, G. (2001). The mitochondrion in apoptosis: how Pandora's box opens. *Nat. Rev. Mol. Cell Biol.* 2, 67–71.
- Zamzami, N., Marchetti, P., Castedo, M., Hirsch, T., Susin, S. A., Masse, B., and Kroemer, G. (1996). Inhibitors of permeability transition interfere with the disruption of the mitochondrial transmembrane potential during apoptosis. *FEBS Lett.* 384, 53–57.
- Zamzami, N., Marchetti, P., Castedo, M., Zanin, C., Vayssiere, J. L., Petit, P. X., and Kroemer, G. (1995). Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J. Exp. Med.* 181, 1661–1672.
- Zhivotovsky, B., and Orrenius, S. (2005). Caspase-2 function in response to DNA damage. *Biochem. Biophys. Res. Commun.* 331, 859–867.