Cell Death and Differentiation (2002) 9, 905–914

www.nature.com/cdd

Mitomycin C induces apoptosis and caspase-8 and -9 processing through a caspase-3 and Fas-independent pathway

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Received 15.10.01; revised 2.4.02; accepted 3.4.02
Edited by RA Knight

Abstract

Caspase-3 activity has been described to be essential for drug-induced apoptosis. Recent results suggest that in addition to its downstream executor function, caspase-3 is also involved in the processing of upstream caspase-8 and -9. To test the absolute requirement for caspase-3, we examined mitomycin C (MMC)-induced apoptosis in the caspase-3 deficient human breast cancer cell line MCF-7. MMC was used as anticancer drug since this agent was preferentially active compared to chemotherapeutic compounds with differing mechanisms of action such as cisplatin, docetaxel, or lovastatin. MMC treatment led to pronounced caspase-8, -9, and -7 processing and early morphological features of apoptosis within 48 h. This could be inhibited by the broad-spectrum caspase inhibitor z-VAD.fmk and to a lesser extent by z-IETD.fmk and z-LEHD.fmk, which have a certain preference for inhibiting caspase-8 and -9, respectively. MMC induced apoptosis in MCF-7 cells was not mediated by the death receptor pathway as demonstrated by experiments using the inhibiting anti-Fas antibody ZB4 and transfections with CrmA, a viral serpin inhibitor of caspase-8, and the dominant negative Fas-associated death domain (FADD-DN). Stable expression with Bcl-2 significantly prevented the processing of caspase-9 but also of caspase-8 and blocked the induction of apoptosis. Thus, we provide evidence that caspase-3 activity is dispensable for MMC-induced apoptosis and for caspase-8 and -9 processing in MCF-7 cells.
Cell Death and Differentiation (2002) **9,** 905–914. doi:10.1038/sj.cdd.4401062

Keywords: caspase-3; caspase-8; caspase-9; Bcl-2; mitomycin C; MCF-7

Abbreviations: MMC, mitomycin C; CrmA, cytokine response modifier A; FADD-DN, dominant negative Fas-associated death domain; z-VAD.fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl keton; DEVD, Ac-Asp-Glu-Val-aspartic acid aldehyde; YVAD, Ac-Tyr-Val-Ala-Asp-chloromethylketone; z-IETD.fmk, z-Ile-Glu(OMe)-Thr-Asp(OMe)-FMK; z-LEHD.fmk, z-Leu-Glu(OMe)-His-Asp-(OMe)-FMK; XIAP, X-linked mammalian inhibitor of apoptosis protein; GFP, green fluorescent protein; IC, inhibitory concentration; WT, wild-type

Introduction

DNA damaging agents are an integral component in the treatment of a large variety of solid and hematological malignancies. DNA damage is a classical inducer of p53 function, which orchestrates apoptosis or DNA repair depending on the cellular background. However, p53 is one of the most frequently mutated genes suggesting that other p53-independent pathways of apoptosis induction are operational for DNA damaging anticancer drugs.[1–3]

It has been proposed that the Fas (CD95) signaling pathway is involved in chemotherapy induced apoptosis.[4–6] Drugs such as doxorubicin or methotrexate have been shown to induce upregulation of Fas receptor as well as Fas ligand expression, thus leading to autocrine and paracrine Fas-dependent apoptosis. Drug-induced apoptosis can be prevented by Fas neutralizing antibodies and a dysfunctional Fas pathway has been shown to correlate with insensitivity to anticancer agents in cell lines. However, various groups were not able to corroborate these findings[7,8] and the importance of the Fas pathway in chemotherapy-induced apoptosis remains controversial.[9–12]

Apoptosis is characterized by a series of stereotype morphological features such as chromatin condensation, nuclear fragmentation, and the appearance of membrane-enclosed apoptotic bodies. These morphological changes are executed by a family of aspartate-specific cysteine proteases (caspases) which can be activated by various apoptogenic signals. In the classical model,[13,14] caspases are divided into initiator caspases (such as caspase-8, -9) and executioner caspases (caspase-3, -6, -7) according to their function and their sequence of activation. The initiator caspases appear to display some specificity according to the type of apoptotic signal. Two main activation cascades for apoptosis induction have been described.[15–17] Fas receptor-ligand interactions use caspase-8 activation to trigger the downstream executioner caspases. An alternative mitochondrial pathway, which is triggered by various anticancer agents, involves activation of caspase-9 upon recruitment to the mitochondria by cytochrome *c* and apoptosis protease activation factor-1 (APAF-1).

More downstream, the initiator caspases lead to the activation of executioner caspases -3, -6, and -7, which in turn cleave specific proteins resulting in the typical hallmarks of apoptosis. Caspase-3 seems to play a central role in chemotherapy-induced apoptosis. It is specifically required for DNA fragmentation leading to the typical apoptotic pattern of DNA laddering.[18,19] Caspase-3 is also necessary for other typical morphological features of apoptotic cell death.[20] In certain systems, such as UV-irradiation and osmotic shock of embryonic stem cells or chemotherapy of mouse embryonic fibroblasts, caspase-3 activity seems even to be an indispensable requirement for the occurrence of apoptosis.[21] In addition, caspase-3 is involved in activating the other effector caspases-6 and -7 as shown in caspase-3 reconstitution experiments of caspase-3 deficient MCF-7 cells.[22]

The temporal and hierarchical order of the activation of the caspase cascade has been questioned by recent observations. Blanc *et al*. have published data to suggest that caspase-3 is not only involved in executing the apoptosis phenotype but is also essential for the processing of upstream procaspase-9 in cisplatin-induced apoptosis.[23] In contrast to this finding, Engels *et al*. found in the same human breast cancer MCF-7 cell line, that anticancer drug-induced caspase-8 processing but not caspase-9 processing was critically dependent on the presence of caspase-3 activity.[24] Another recent paper has corroborated downstream caspase-8 activation by caspase-3 in a B-cell lymphoma system.[25] These results suggest that downstream executioner caspases can amplify the function of upstream initiator caspases. Additional amplifying loops might be in place between the classical Fas signal transduction pathway and the mitochondrial/cytochrome *c*/APAF-1 pathway of apoptosis induction.

We therefore asked the question, how these pathways interact in anticancer drug-induced apoptosis in the well described system of the human breast cancer cell line MCF-7. Mitomycin C (MMC) was used as anticancer drug since this agent was preferentially active in MCF-7 cells compared to chemotherapeutic compounds with differing mechanisms of action such as cisplatin, docetaxel, or lovastatin. MCF-7 cells lack caspase-3 due to a 47-base pair deletion within exon 3 of the CASP-3 gene,[20] which allowed us to examine whether caspase-3 is central for the feedback activation of initiator caspases -8 and -9 for executioning apoptosis. Despite recent findings to show otherwise, we found caspase-8 and -9 activation in the absence of caspase-3 activity.

Results

Induction of apoptosis with MMC in the human breast cancer cell line MCF-7

To determine the cytotoxic effect of various anticancer drugs with different mechanisms of action in MCF-7 cells, the dose response over 5 days was assessed using the Trypan Blue exclusion assay (Figure 1). The viability of MCF-7 cells was decreased by 87% after 72 h treatment with 5 μg/ml MMC. In comparison, cisplatin led to a 31%, taxotere to a 25%, and lovastatin to a 17% decrease in viability, respectively. Thus, since MMC has shown the most potent cytotoxic activity, this drug was used to induce apoptosis in further experiments.

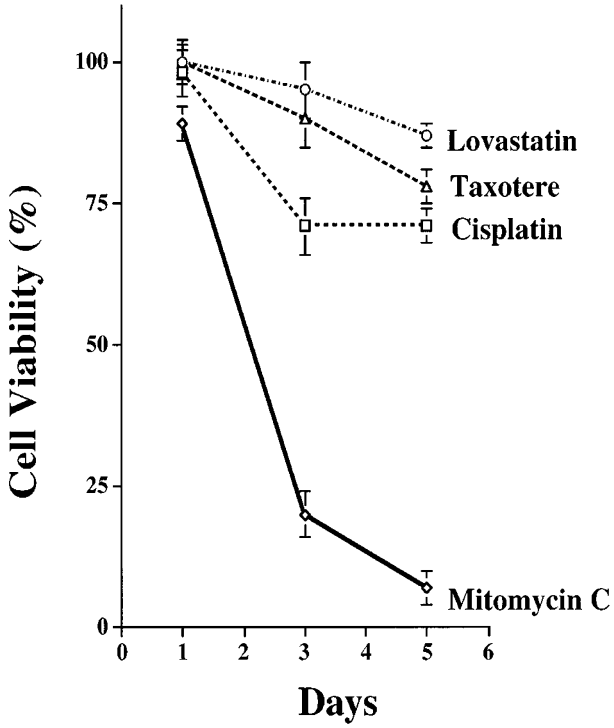

Figure 1 Cytotoxic effect of various anticancer drugs in MCF-7 cells. MCF-7 cells were treated with 5 μg/ml MMC, 10 μg/ml taxotere, 10 μg/ml cisplatin, and 10 μM lovastatin for the indicated time periods. Data are the mean of triplicates; similar results were obtained in three separate experiments. Percentage of untreated controls at the same time point

Apoptotic cell death is defined by the occurrence of a stereotype phenotype including cell shrinkage, chromatin condensation and nuclear fragmentation. However, Johnson *et al*. have identified an early nuclear morphological change in caspase-3 null MCF-7 cells, which precedes chromatin condensation during chemical-induced apoptosis.[26] To verify, that decrease of viability correlated with the induction of apoptotic cell death in this system, MMC treated cells were stained with the DNA intercalating Hoechst 33342 dye. This experiment showed that 82% of MMC treated MCF-7 cells underwent nuclear condensation (Figure 2a). Since apoptosis is executed by caspases, we used the promiscuous cell membrane permeable caspase inhibitor z-VAD.fmk as an apoptosis specific blocking agent. As shown in (Figure 2b,c), apoptotic cell death could be largely prevented by co-administering z-VAD.fmk with MMC. To assess whether the different treatments did affect viability rather than mere apoptotic phenomena, we performed clonogenic growth assays. The results of these experiments showed that adding z-VAD.fmk to MMC treated cells did significantly increase clonogenic survival in addition to preventing apoptotic morphology (Figure 2d, Table 1). These results confirm that the cytotoxic activity of MMC results mainly from the induction of the apoptosis machinery, which is dependent on the activation of the caspase cascade.

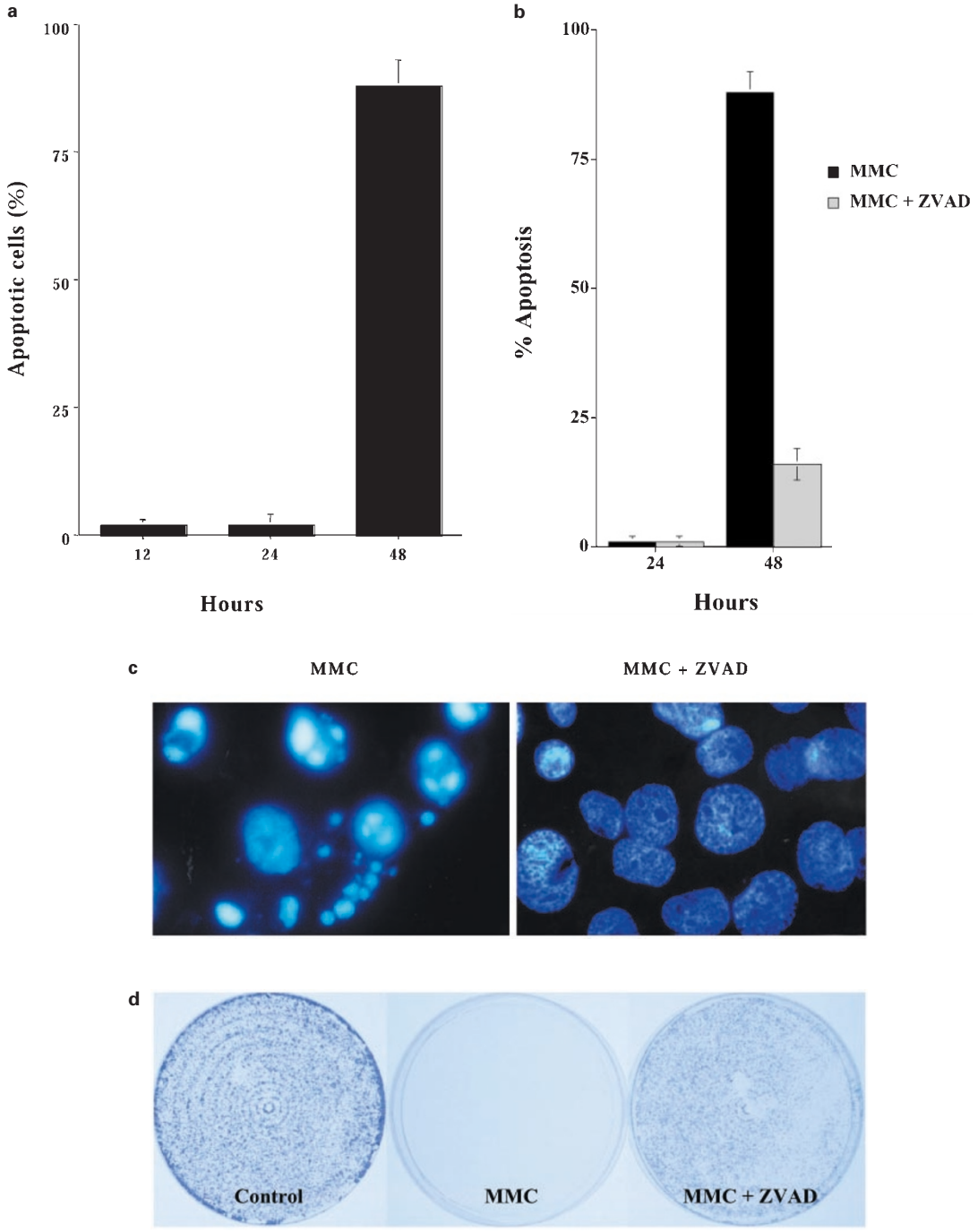

Figure 2 Drug-induced apoptosis in MCF-7 cells. (**a**) MCF-7 cells were treated with 5 μg/ml MMC for indicated periods and stained with Hoechst 33342. Then cells were counted under the fluorescent microscope as described in Material and Methods. Percentage of untreated controls. (**b**) MCF-7 cells were treated with 5 μg/ml MMC in the absence or presence of 100 μM z-VAD.fmk for indicated periods and stained with Hoechst 33342. Apoptotic cells were quantified as described in Materials and Methods. Data are the mean of triplicates; similar results were obtained in three separate experiments. Percentage of untreated controls (**c**) Hoechst staining illustrating nuclear condensation characteristic of apoptosis after MMC treatment and the blocking effect of co-administering z-VAD.fmk. (**d**) Colony formation assay. After treatment with 5 μg/ml of MMC in the presence or absence of z-VAD for 48 h as described in Material and Methods, colonies were stained and counted. Representative culture dishes of the same experiment are depicted

Caspase-3 independent caspase-8, -9 and -7 processing with MMC treatment

The initiator caspases -8 and -9 are activated by proapoptotic signals such as Fas receptor stimulation or cytotoxic agents respectively. There is also recent evidence that the effector caspase-3 itself is involved in caspase-8 and -9 activation.[23–25] This led us to test caspase-8 and -9 activation in MCF-7 cells, which are devoid of caspase-3

Table 1 Impact of transfection with various apoptosis inhibitory proteins, Bcl-2, or treatment with various caspase inhibitory peptides on clonogenic survival of MCF-7 cells treated with MMC for 48 h. MMC was used at the IC^{80} and IC^{50} concentrations for this cell line

MMC	Parental (%)[a,b]	pcDNA3 (%)	CrmA (%)	XIAP (%)	FADD-DN (%)	Bcl-2 (%)	pCdeltaj-SV-2 (%)	z-IETD (%)	z-LEHD (%)	z-VAD (%)
IC^{80}	0	0	0	0	0	48	0	0	0	64
IC^{50}	21	18	23	17	18	67	22	22	21	85

[a]Colony-forming units are indicated as percentage of the respective untreated control. [b]Numbers depicted represent the mean of three independent experiments using triplicates

activity. Since chemotherapy typically leads to caspase-9 activation by engaging cytochrome *c* and APAF-1 at the mitochondrial membrane,[27] we examined caspase-9 activation by MMC in the MCF-7 cells. Caspase-9 activation was assessed by verifying the cleaving of the caspase-9 49-kDa proform to the characteristic 35 kD fragment. This 35 kD fragment appeared within 24 h of MMC treatment as detected by Western blotting (Figure 3a) suggesting a role of caspase-9 activation for MMC induced apoptosis in MCF-7 cells. Caspase-8, which is the most apical caspase in the Fas death signaling pathway was also activated by MMC as shown by the appearance of the typical p41/42 fragment and a lower size fragment of around 26 kD (Figure 3a). However, this caspase-8 processing appeared only after 36 h suggesting an activation independent or downstream of caspase-9. Caspase-7, which is another effector caspase, was also activated by MMC treatment and processed into the typical p20 fragment within 36 h (Figure 3a). Procaspase-6 was upregulated by MMC treatment but displayed no evidence of activation (Figure 3a). Figure 3b shows the pattern of caspase-8 and -9 activation upon treatment with various anticancer agents with differing mechanism of action. MMC led to a preferential cleaving of caspase-8.

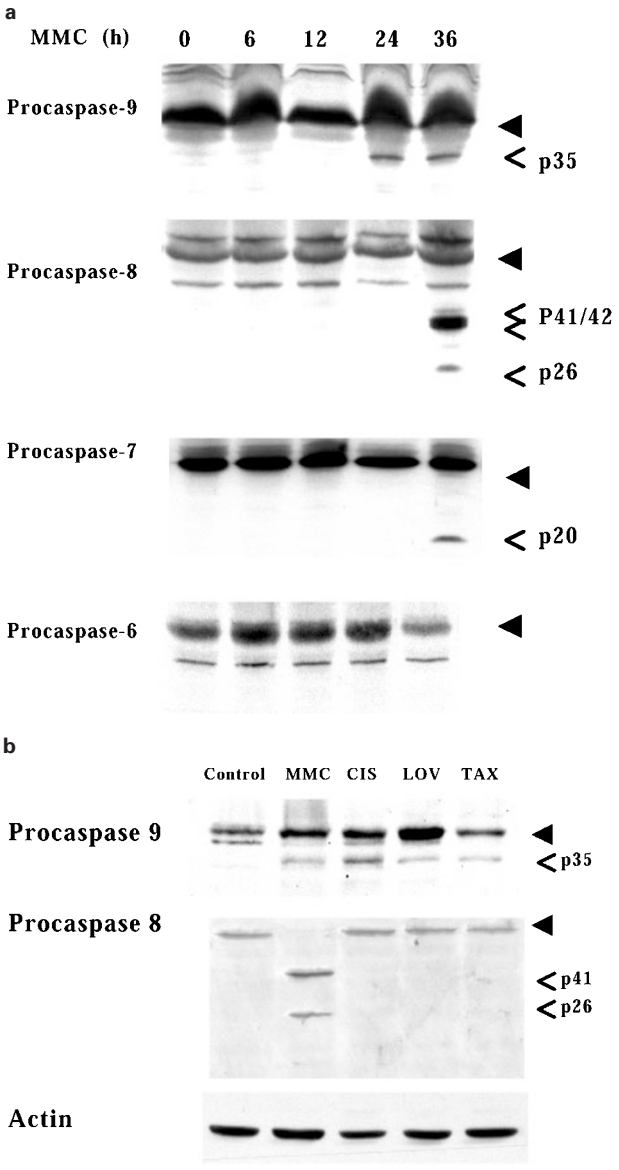

Figure 3 Kinetics of caspase-3 independent caspase-9, -8, -7 and -6 processing. (**a**) MCF-7 cells were treated with 5 μg/ml MMC for indicated time periods and cellular proteins were resolved by SDS–PAGE. Caspase-activity was detected by cleavage of caspase-7, -8, -9 at indicated times. Filled arrowheads indicate the uncleaved and open arrowheads the cleaved form of the respective protein. (**b**) MCF-7 cells were treated with 5 μg/ml MMC, 10 μg/ml taxotere, 10 μg/ml cisplatin, and 10 μM lovastatin for 48 h and processed as described in (**a**)

Caspase-8 processing after MMC treatment is not dependent upon Fas activation

Caspase-8 is the classical initiator caspase of the Fas pathway, which is stimulated by Fas receptor/ligand interactions. The Fas pathway has also been shown to contribute to chemotherapy-induced apoptosis in various cellular systems.[4–6] Since Fas receptor upregulation is associated with drug-induced activation of the Fas death signaling pathway, Fas receptor expression changes were assessed by immunoblotting. MMC treatment did not lead to an induction of the Fas receptor (Figure 4a). To further explore the involvement of the Fas pathway in drug-induced apoptosis, cells were cotreated with MMC and ZB4, an inhibiting anti-Fas antibody that blocks Fas mediated killing.[9] This antibody failed to prevent apoptosis (Figure 4b). In addition, MCF-7 cells were transfected with the adapter molecule Fas associated death domain dominant negative (FADD-DN;[28,29]) and with CrmA.[30] Both did neither protect from MMC-induced apoptosis (Figure 5d and Table 1) nor inhibit caspase-8 or -9 activation (Figure 4c). All these experiments supported the notion

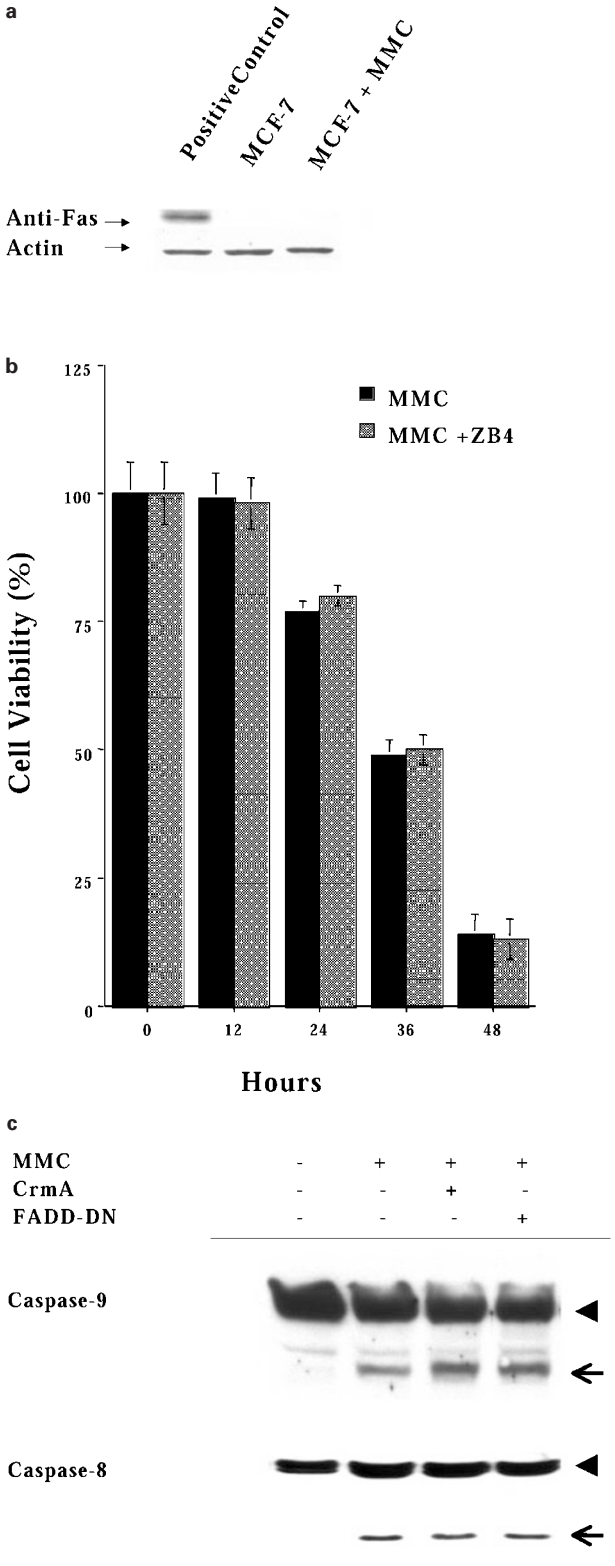

Figure 4 Fas-independent caspase-8 processing after MMC. (**a**) Fas receptor was detected by Western blot analysis. The Jurkat cell line served as a positive control. Expression of actin was used to control equal protein loading. (**b**) MCF-7 cells were treated with 5 μg/ml MMC in the absence or presence of 1 μg/ml ZB4 for indicated periods and stained with Hoechst 33342 to quantify apoptosis. Data are the mean of triplicates; similar results were obtained in three separate experiments. Percentage of untreated controls. (**c**) MCF-7 cells transfected with CrmA WT, FADD-DN, or an empty vector were treated with 5 μg/ml MMC and cellular proteins were resolved by SDS – PAGE. Filled arrowheads indicate the uncleaved and open arrowheads the cleaved form of the respective protein

that a death receptor pathway was not involved in MMC-induced caspase-8 activation and apoptosis in this system.

Impact of various caspase inhibitory peptides and apoptosis inhibiting proteins on MMC induced apoptosis

Alternative activators had to contribute to caspase-8 activation because of the fact that we found no evidence for the involvement of the Fas receptor or caspase-3 in MCF-7 cells. Since processing of caspase-9 occurred earlier than processing of caspase-8 in our system, we looked at the possibility of caspase-9 dependent processing of caspase-8. For this purpose, we used z-IETD.fmk, an inhibitor of caspase-8, and z-LEHD.fmk, an inhibitor of caspase-9, which have been described to be specific for the respective caspase.[31] MMC induced caspase-8 processing was completely blocked by z-IETD.fmk without effect on the processing of caspase-9 (Figure 5a). The caspase-9 inhibitor z-LEHD.fmk, on the other hand, had no effect on the accumulation of the cleaved caspase-9 itself but led to a near complete inhibition of caspase-8 activation. This lacking specificity of z-LEHD.fmk has recently been corroborated by other investigators.[32] The caspase-8 inhibitor z-IETD.fmk had a stronger blocking effect than z-LEHD.fmk on MMC induced apoptosis (Figure 5b,c). However, for both inhibitors and also less toxic concentrations of MMC, this effect was less than 30% limiting the strength of these results concerning conclusions on the impact of the respective caspase. This interpretation is corroborated by the results of the clonogenic growth assays, where both caspase inhibitors had no effect on clonogenic survival in contrast to the potent impact of z-VAD.fmk (Table 1). DEVD, which inhibits caspases-3 family members,[33] YVAD, which inhibits caspase-1 family members[34] and the apoptosis inhibitory protein XIAP[35] did all not prevent MMC-induced apoptosis (Figure 5d and Table 1).

Overexpression of Bcl-2 protects MCF-7 cells from MMC-induced apoptosis and processing of caspase-8 and -9

Bcl-2 inhibits release of cytochrome *c* from mitochondria preventing the activation of caspase-9 and apoptosis.[36] We determined if the processing of caspase-9 or other caspases, which were activated by MMC treatment in MCF-7 cells, was inhibited by enforced Bcl-2 expression. Both control cells, which had been transfected with an empty vector (MCF-7va) and two clones of cells overexpressing Bcl-2 (MCF-7/Bcl-2) (Figure 5f) were treated with MMC and apoptosis was quantified by Hoechst staining (Figure 6a). Transfection with Bcl-2 inhibited MMC induced apoptosis by 65%. Overexpressing of Bcl-2 completely blocked caspase-9 activation

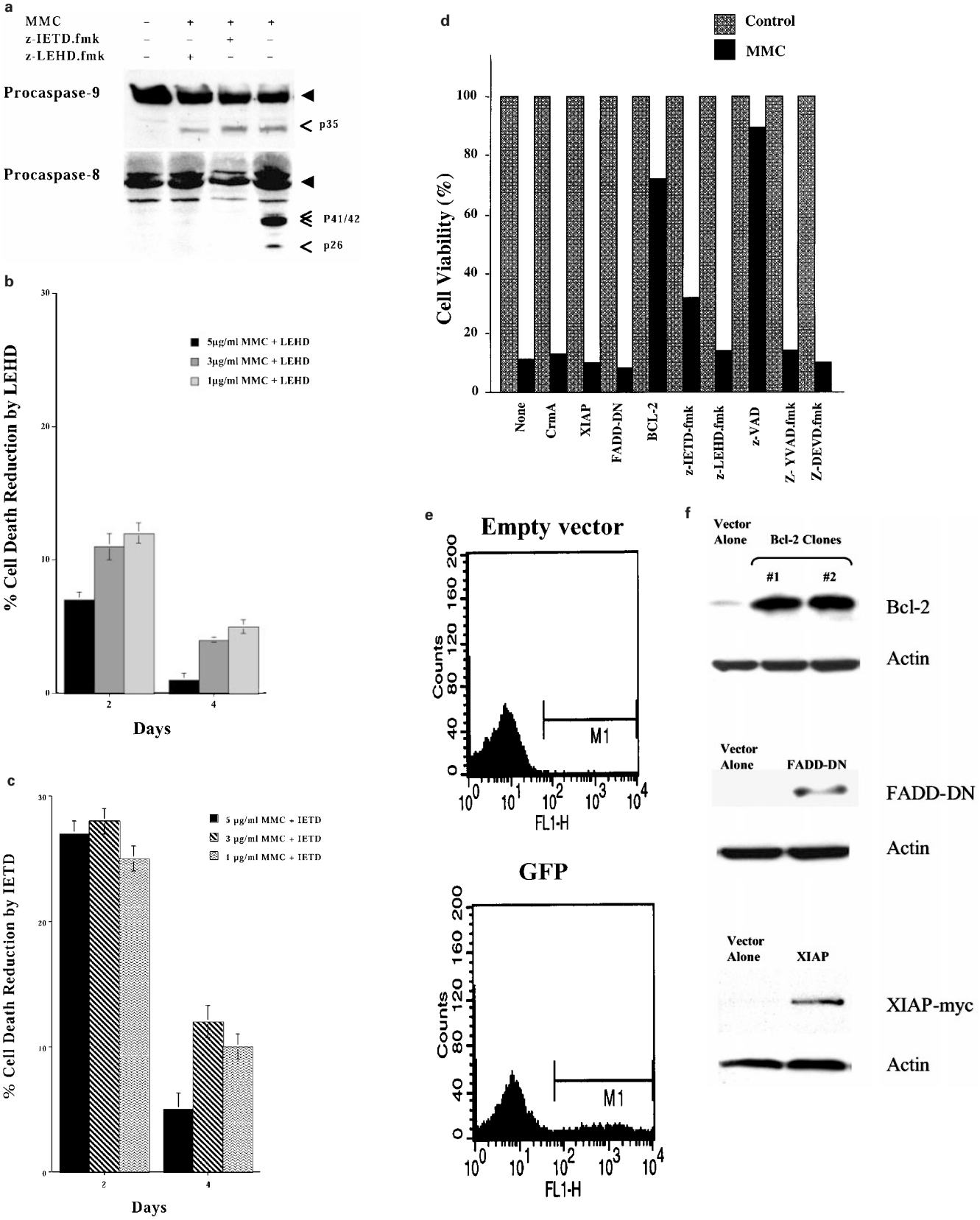

Figure 5 Impact of caspase inhibitors on relative caspase-8 and -9 processing after MMC treatment. (**a**) MCF-7 cells were treated for 36 h with 5 μg/ml MMC in the absence and presence of z-IETD.fmk (caspase-8 inhibitor) or z-LEHD.fmk (caspase-9 inhibitor). Filled arrowheads indicate the uncleaved and open arrowheads the cleaved form of the respective protein. (**b**)+(**c**) Cytotoxic effect of different MMC concentrations for 48 and 96 h in the presence or absence of z-LEHD or z-IETD

and to a lesser extent caspase-8 processing after MMC treatment (Figure 6b).

Discussion

The results of these experiments add further evidence for the complexity of the interplay of different caspases in drug induced apoptosis. In accordance with other recently published work,[8,24,25,37] we demonstrate that the role of caspase-8 is not restricted to death receptor induced apoptosis, but is also crucially involved in chemotherapy induced cell death. Although different reports demonstrate that cytotoxic drugs induce apoptosis by triggering Fas signalling, the conflicting data from the literature suggest that the involvement of this pathway is dependent on the cellular background. We found no evidence for the involvement of the Fas death pathway in MMC-induced apoptosis in MCF-7 cells. This is in line with other reports on a disabled Fas pathway in this cell line,[38] which is not induced by DNA damaging agents such as MMC.[39] Our results suggest a purely intracellular mechanism of caspase-8 activation analogous to the activation of caspase-9 within the apoptosome.

Various lines of evidence suggest that caspase-8 was activated downstream from caspase-9 in our system. Firstly, the analysis of the temporal activation pattern revealed that the processing of caspase-9 was initiated before caspase-8 processing. Secondly, the preferential caspase-9 peptide inhibitor z-LEHD.fmk had a pronounced blocking effect on caspase-8 processing. Thirdly, Bcl-2 blocked caspase-9 but also caspase-8 cleavage despite the fact, that Bcl-2 has shown not to inhibit Fas induced caspase-9 signaling.[40] However, Fas-independent caspase-8 activation might still be blocked by Bcl-2. An alternative hypothesis to the sequential activation of caspase-9 and -8 would be that caspase-8 is activated independently or in parallel with caspase-9. This is in line with other recent studies, which have shown that caspase-8 activation in response to cytotoxic drugs is mediated by mitochondria rather than the Fas pathway.[37,41] In various systems such as the lung cancer cell line H460 or neuroblastoma cells, a scenario has been suggested, that APAF-1 together with cytochrome *c* and caspase-8 could form an alternative apoptosomal complex.[37,41] Also, it has been described that caspase-3 activates caspase-8 via

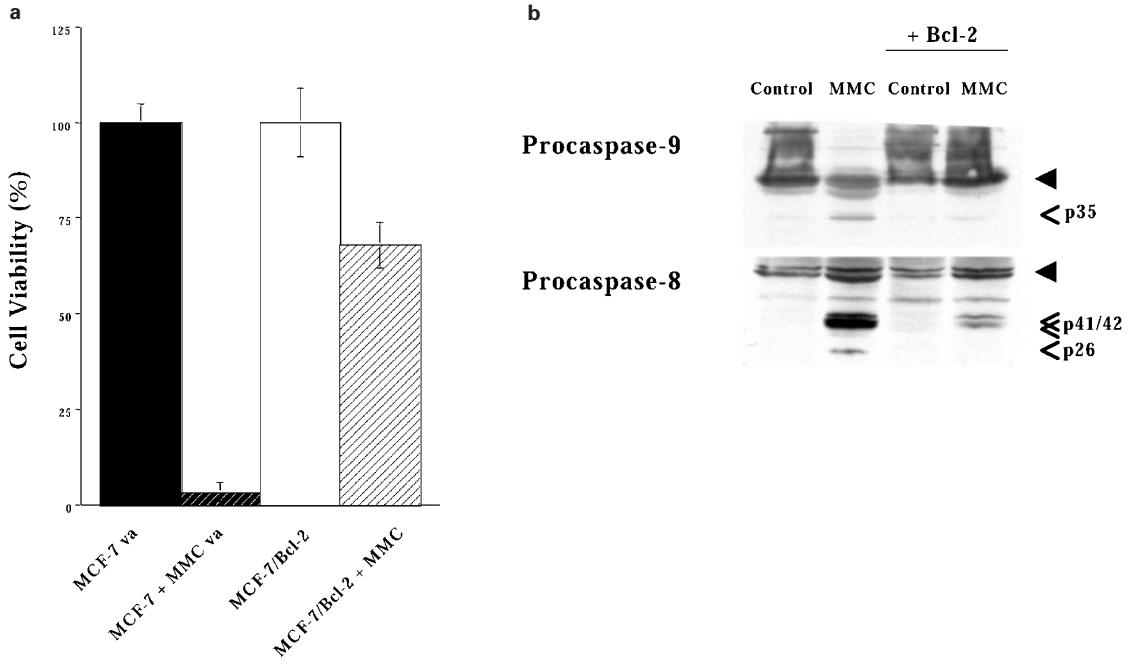

Figure 6 Over-expression of Bcl-2 protects MCF-7 cells from MMC-induced apoptosis and inhibits processing of caspase-8 and -9. (**a**) MCF-7 cells were stably transfected with either pCdeltaj-SV-2 (vector-only) or pCdeltaj-bcl-2 (bcl-2) plasmid. Cells were treated with 5 μg/ml MMC for 48 h and stained with Hoechst 33342 to quantify apoptosis. Data are the mean of triplicates; similar results were obtained in three separate experiments. (**b**) Immunoblotting for caspase-8, -9 with the conditions described under (**a**). Filled arrowheads indicate the uncleaved and open arrowheads the cleaved form of the respective protein

as described in Material and Methods. MMC is used at the IC^{80} (5 μg/ml), IC^{50} (3 μg/ml) and IC^{30} (1 μg/ml) concentrations. Data are the mean of triplicates; similar results were obtained in three separate experiments. (**d**) Effects of various inhibitors of apoptosis, caspase inhibitory peptides, and Bcl-2 on the viability of MMC treated MCF-7 cells. Cells were transfected with the respective inhibitors of apoptosis or exposed to the respective caspase inhibitory peptide and treated with 5 μg/ml of MMC for 48 h. Hoechst 33342 staining was used to quantify apoptosis. (**e**) Results of FACS analysis demonstrating co-transfection efficiency for GFP in CrmA transfectants. M1 area=GFP (CrmA) co-transfected cells. (**f**) Western blot analysis confirming the ectopic expression of XIAP-*myc*, FADD-DN, or Bcl-2 (two different clones) compared with empty vector transfected cells

caspase-6.[42] This is consistent with the lack of caspase-6 activation in our system.

A recent report places caspase-8 cleavage downstream of caspase-3 activation and describes functionally relevant block of caspase-8 processing, if B-lymphoid cells are depleted of caspase-3.[25] Although our experiments do not exclude the possibility, that caspase-8 would have been more extensively or rapidly cleaved in the presence of active caspase-3, we found pronounced and functionally relevant caspase-8 processing induced by MMC treatment in the MCF-7 human breast cancer cell line, which is devoid of caspase-3 protein.[20] Our results also show, that caspase-9 processing is not dependent on caspase-3 activity in contrast to the conclusions of a recent paper by Blanc *et al*., which postulates an essential function of caspase-3 for caspase-9 processing.[23] Thus, other caspases can clearly replace the lack of caspase-3 function in MCF-7 cells. The mutual activation capacity of different caspases seems to be more pronounced than formerly appreciated. Although specific caspases have their preferential activation partners, this partnership seems not to be exclusive and might be influenced by the expression pattern of the various caspases and interacting molecules in the respective cell.

Two groups have recently reported their work on the functional impact of restoring caspase-3 activity by transfection in MCF-7 cells.[22,43] Both found that restoring caspase-3 activity translated into a significantly higher activity of the anticancer drugs etoposide, doxorubicin, epirubicin and taxol. In contrast, even high drug concentrations led to only sporadic islands of cell death in caspase-3 defective cells suggesting a crucial role for this enzyme in drug-induced apoptosis.[22] Doxorubicin, epirubicin, and etoposide are inhibitors of topoisomerase II,[44,45] while taxol interacts with tubulin metabolism.[46] It can be speculated, that drugs with other mechanisms of action might be less dependent on caspase-3 activity for apoptosis induction. In support of this notion, we found a 40% higher cytotoxic activity of MMC as used in our experiments in comparison to doxorubicin at the concentrations used by Yang *et al*. in wild-type MCF-7 cells (Pirnia *et al*., unpublished results). MMC led to an exclusive activation of caspase-8 but not of caspase-9 in comparison to various other drugs with differing mechanisms of action at concentrations formerly established as equitoxic in the NCI/ADR-RES cell line (Figure 3b).[47] This suggests a caspase-9 independent caspase-8 activation, which correlates well with the preferential cytotoxic activity of MMC in this system. Since MMC as a DNA damaging agent is a very strong activator of p53 function compared to taxol and other anticancer drugs (Pirnia *et al*., unpublished results),[48,49] our findings could be interpreted in the context of recent data, which show p53 to be crucially involved in the transcriptional activation of APAF-1.[50] Thus, the potent p53 stimulatory effect of MMC resulting in transcriptional activation of death pathway components may override the lack of caspase-3 function.

The promising observation by Friedrich and Wieder *et al*. that procaspase-3 overexpression restores sensitivity for drug-induced apoptosis without affecting background apoptosis provides hope that the therapeutic modulation of caspase-3 activity is not toxic by itself.[43] Thus, replacing or enforcing the function of individual caspases might serve as a therapeutic tool for increasing the efficacy of cytotoxic anticancer agents. In addition, our results suggest that the caspase activation cascade is not an uniform machinery, where one missing component leads to a functional shutdown, but rather a redundant system. The type of inducing stimulus might override the lack of an individual caspase as shown here for MMC in the case of lacking caspase-3 activity.

Materials and Methods

Cell culture, anticancer drugs, expression constructs, and transfections

The human mammary carcinoma cell line MCF-7 (obtained from ATCC, Rockville, MD, USA) was grown at 37°C in RPMI 1640 medium containing 10% (v v) heat-inactivated fetal bovine serum, 100 units/ml penicillin G, 100 mg/ml streptomycin, and 2 mM glutamine in an atmosphere containing 5% (v v) CO_2). Taxotere (40 mg/ml) was purchased from Rhône-Poulenc Rorer (Thalwil, Switzerland), cisplatin (0.5 mg/ml) and mitomycin C (0.5 mg/ml) from Bristol-Myers Squibb (Baar, Switzerland) and lovastatin was a kind gift from Merck, Sharp and Dohme Research Pharmaceuticals Co. (Syracuse, NY, USA). Lovastatin (10 mM) was converted from the inactive lactone form into its active dihydroxy open acid form as described.[51] MCF-7 human breast cancer cells were transfected by the calcium phosphate method with either plasmid pCdeltaj-SV-2 (vector-only) or pCdeltaj-bcl-2 (bcl-2) that had been generously provided by Y. Tsujimoto.[52] Following transfection, the cells were selected in 1 g/l G418 until individual colonies appeared. Clones were collected separately and analyzed for Bcl-2 expression by Western blot (Figure 5f). The pcDNA3CrmA wild-type was a generous gift from M. Tewari[53] and pDNA3 XIAP (*myc*-tagged) from Q. Deveraux.[54] The AU1 FADD Dominant Negative pcDNA3 was a generous gift from V. Dixit.[55] The pEGFP-N1 plasmid (GFP) was purchased from BD CLONTECH (Basel, Switzerland). MCF-7 cells were transfected using TransFast™ (Promega Corporation, Basel, Switzerland) according to the manufacturer's protocol. Transfection efficiency assessed by co-transfection with a green fluorescent protein (GFP) expression vector was between 30–50%. The Western blot confirming the ectopic expression of XIAP-*myc*, FADD-DN, and Bcl-2 is shown in Figure 5f. Since the CrmA plasmid was non-tagged, transfection efficiency was assessed using FACS quantification of GFP (Figure 5e). After 36 h, GFP positive cells were quantified by flow cytometry (Becton Dickinson, CA, USA).

Caspase inhibitors

The caspase inhibitory peptides benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl keton (z-VAD.fmk), Ac-Asp-Glu-Val-aspartic acid aldehyde (DEVD), and Ac-Tyr-Val-Ala-Asp-chloromethylketone (YVAD) was obtained from Bachem (Bubendorf, Switzerland). The inhibitors z-lle-Glu(OMe)-Thr-Asp(OMe)-FMK (z-IETD.fmk) and z-Leu-Glu(OMe)-His-Asp-(OMe)-FMK (z-LEHD.fmk) were purchased from Enzyme System Products (CA, USA). Caspase inhibitors were used at a concentration of 40 μM for (z-IETD.fmk and z-LEHD.fmk) and 100 μM for (z-VAD.fmk), DEVD, and YVAD 2 h prior to chemotherapy exposure.

Apoptosis assay

Cells were detached with 5 mM EDTA, cytospinned onto glass, and fixed with 3.7% formaldehyde for 15 min at RT. Staining was performed with 1 μg/ml Hoechst 33342 in PBS. Coverslips

were mounted on the slides with Slowfade (Molecular Probes, Oregon, USA). The cells were examined on a Leica DMRB photomicroscope using a 100 × magnification lens. To quantify apoptotic cells, 300 nuclei were counted for each experimental condition.

Cell viability assay

Cell viability was determined by Trypan blue exclusion. Fifty per cent of cell suspension was mixed with 50% of Trypan blue isotonic solution (0.2%; w v), and cell viability was determined on a haemocytometer under a microscope.

Clonogenic assays

MCF-7 cells were seeded in triplicate into six-well plates at a concentration of 300 cells per well. After 24 h, MMC were added at a final concentration corresponding to the IC^{80}, IC^{50} and IC^{30} for 48 h. After drug removal, cells were washed twice with PBS and allowed to proliferate in fresh medium. Colonies were counted when they reached the size of 50 – 100 cells, after staining with 0.1% crystal violet in 0.9% saline for 30 min at room temperature. The number of colony-forming units in treated cultures was expressed as the percentage on untreated controls. The respective inhibitors were added 2 h prior to MMC treatment.

Western blot analysis

Cells were collected and lysed with TNN lysis buffer (40 mM Tris, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 0.1 mM sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptine, and 100 μg/ml PMFS) at 4°C for 1 h. Lysates were spun at 14 000 r.p.m. for 20 min to remove cellular debris. Protein concentration was determined with the BCA reagent (Pierce, IL, USA). Equal amounts of protein were subjected to electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by electroblotting. The blots were blocked, probed for 1 h with the primary antibody, washed with 10 mM Tris, pH 7.5, 50 mM NaCl and 2.5 mM EDTA, and visualized using a secondary goat antimouse or anti-rabbit antibody conjugated with horseradish peroxidase. Chemiluminescence was performed with ECL (Amersham Life Science, Dübendorf, Switzerland). The following primary antibodies were used: caspase-8 p20, (c-20, polyclonal), caspase-3, Fas (C-20)-G (polyclonal), c-Myc and Bcl-2(100) (monoclonal) from Santa Cruz Biotechnology (CA, USA) and caspase-6, -7 monoclonal antibody were purchased from Pharmingen International (CA, USA). Caspase-9 and Anti-FADD (monoclonal) from MBL (Naka-ku Naagoya, Japan).

Acknowledgements

This work was supported by a grant from the Swiss Cancer League.

References

1. Borner MM, Myers CE, Sartor O, Sei Y, Toko T, Trepel JB and Schneider E(1995) Drug-induced apoptosis is not necessarily dependent on macromolecular synthesis or proliferation in the p53-negative human prostate cancer cell line PC-3. Cancer Res. 55: 2122 – 2128
2. Ferreira CG, Tolis C and Giaccone G (1999) p53 and chemosensitivity. Ann. Oncol. 10: 1011 – 1021
3. Strasser A, Harris AW, Jacks T and Cory S (1994) DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2. Cell 79: 329 – 339
4. Muller M, Strand S, Hug H, Heinemann EM, Walczak H, Hofmann WJ, Stremmel W, Krammer PH, Galle PR (1997) Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. J. Clin. Invest. 99: 403 – 413
5. Fulda S, Sieverts H, Friesen C, Herr I and Debatin KM (1997) The CD95 (APO-1/Fas) system mediates drug-induced apoptosis in neuroblastoma cells. Cancer Res. 57: 3823 – 3829
6. Friesen C, Herr I, Krammer PH and Debatin KM (1996) Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nat. Med. 2: 574 – 577
7. Newton K and Strasser A (2000) Ionizing radiation and chemotherapeutic drugs induce apoptosis in lymphocytes in the absence of Fas or FADD/MORT1 signaling. Implications for cancer therapy. J. Exp. Med. 191: 195 – 200
8. Wesselborg S, Engels IH, 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
9. Eischen CM, Kottke TJ, Martins LM, Basi GS, Tung JS, Earnshaw WC, Leibson PJ and Kaufmann SH (1997) Comparison of apoptosis in wild-type and Fas-resistant cells: chemotherapy-induced apoptosis is not dependent on Fas/Fas ligand interactions. Blood 90: 935 – 943
10. Villunger A, Egle A, Kos M, Hartmann BL, Geley S, Kofler R and Greil R (1997) Drug-induced apoptosis is associated with enhanced Fas (Apo-1/CD95) ligand expression but occurs independently of Fas (Apo-1/CD95) signaling in human T-acute lymphatic leukemia cells. Cancer Res. 57: 3331 – 3334
11. Tolomeo M, Dusonchet L, Meli M, Grimaudo S, D'Alessandro N, Papoff G, Ruberti G and Rausa L (1998) The CD95/CD95 ligand system is not the major effector in anticancer drug-mediated apoptosis. Cell Death Differ. 5: 735 – 742
12. Fulda S, Friesen C, Los M, Scaffidi C, Mier W, Benedict M, Nunez G, Krammer PH, Peter ME, Debatin KM (1997) Betulinic acid triggers CD95 (APO-1/Fas)- and p53-independent apoptosis via activation of caspases in neuroectodermal tumors. Cancer Res. 57: 4956 – 4964
13. Thornberry NA and Lazebnik Y (1998) Caspases: enemies within. Science 281: 1312 – 1316
14. Strasser A, Harris AW, Huang DC, Krammer PH and Cory S (1995) Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. EMBO J. 14: 6136 – 6147
15. Sun XM, MacFarlane M, Zhuang J, Wolf BB, Green DR and Cohen GM (1999) Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. J. Biol. Chem. 274: 5053 – 5060
16. Earnshaw WC, Martins LM and Kaufmann SH (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu. Rev. Biochem. 68: 383 – 424
17. Budihardjo I, Oliver H, Lutter M, Luo X and Wang X (1999) Biochemical pathways of caspase activation during apoptosis. Annu. Rev. Cell Dev. Biol. 15: 269 – 290
18. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A and Nagata S (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391: 43 – 50
19. Liu X, Li P, Widlak P, Zou H, Luo X, Garrard WT and Wang X (1998) The 40-kDa subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis. Proc. Natl. Acad. Sci. USA 95: 8461 – 8466
20. Janicke RU, Sprengart ML, Wati MR and Porter AG (1998) Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J. Biol. Chem. 273: 9357 – 9360
21. Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D, Hakem A, McCurrach M, Khoo W, Kaufman SA, Senaldi G, Howard T, Lowe SW and Mak TW (1998) Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev. 12: 806 – 819
22. Yang XH, Sladek TL, Liu X, Butler BR, Forelich CJ and Thor AD (2001) Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to doxorubicin- and etoposide-induced apoptosis. Cancer Res. 61: 348 – 354
23. Blanc C, Deveraux QL, Krajewski S, Janicke RU, Porter AG, Reed JC, Jaggi R and Marti A (2000) Caspase-3 is essential for procaspase-9 processing and cisplatin-induced apoptosis of MCF-7 breast cancer cells. Cancer Res. 60: 4386 – 4390

24. Engels IH, Stepczynska A, Stroh C, Lauber K, Berg C, Schwenzer R, Wajant H, Janicke RU, Porter AG, Belka C, Gregor M, Schulze-Osthoff K, Wesselborg S (2000) Caspase-8/FLICE functions as an executioner caspase in anticancer drug-induced apoptosis. Oncogene 19: 4563 – 4573
25. Wieder T, Essmann F, Prokop A, Schmelz K, Schulze-Osthoff K, Beyaert R, Dorken B and Daniel PT (2001) Activation of caspase-8 in drug-induced apoptosis of B-lymphoid cells is independent of CD95/Fas receptor-ligand interaction and occurs downstream of caspase-3. Blood 97: 1378 – 1387
26. Johnson VL, Ko SCW, Holmstrom TH, Eriksson JE and Chow SC (2000) Effector caspases are dispensable for the early nuclear morphological changes during chemical-induced apoptosis. J. Cell. Science 113: 2941 – 2953
27. Zou H, Li Y, Liu X and Wang X (1999) An APAF-1 cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J. Biol. Chem. 274: 11549 – 11556
28. Newton K, Harris AW, Bath ML, Smith KG and Strasser A (1998) A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes. EMBO J. 17: 706 – 718
29. Tschopp J, Irmler M and Thome M (1998) Inhibition of fas death signals by FLIPs. Curr. Opin. Immunol. 10: 552 – 558
30. Zhou Q, Snipas S, Orth K, Muzio M, Dixit VM and Salvesen GS (1997) Target protease specificity of the viral serpin CrmA. Analysis of five caspases. J. Biol. Chem. 272: 7797 – 7800
31. Kovar H, Jug G, Printz D, Bartl S, Schmid G and Wesierska-Gadek J (2000) Characterization of distinct consecutive phases in non-genotoxic p53-induced apoptosis of Ewing tumor cells and the rate-limiting role of caspase 8. Oncogene 19: 4096 – 4107
32. Jiang C, Wang Z, Ganther H and Lu J (2001) Caspases as key executors of methyl selenium-induced apoptosis (anoikis) of DU-145 prostate cancer cells. Cancer Res. 61: 3062 – 3070
33. Srinivasula SM, Ahmad M, Fernandes-Alnemri T and Alnemri ES (1998) Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol. Cell. 1: 949 – 957
34. Nicholson DW and Thornberry NA (1997) Caspases: killer proteases. Trends Biochem. Sci. 22: 299 – 306
35. Deveraux QL and Reed JC (1999) IAP family proteins – suppressors of apoptosis. Genes Dev. 13: 239 – 252
36. Green DR and Reed JC (1998) Mitochondria and apoptosis. Science 281: 1309 – 1312
37. Ferreira CG, Span SW, Peters GJ, Kruyt FA and Giaccone G (2000) Chemotherapy triggers apoptosis in a caspase-8-dependent and mitochondria-controlled manner in the non-small cell lung cancer cell line NCI-H460. Cancer Res. 60: 7133 – 7141
38. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH and Peter ME (1998) Two CD95 (APO-1/Fas) signaling pathways. EMBO J. 17: 1675 – 1687
39. Muller M, Wilder S, Bannasch D, Israeli D, Lehlbach K, Li-Webber M, Friedman SL, Galle PR, Stremmel W, Oren M and Krammer PH (1998) p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. J. Exp. Med. 188: 2033 – 2045
40. Huang DC, Hahne M, Schroeter M, Frei K, Fontana A, Villunger A, Newton K, Tschopp J and Strasser A (1999) Activation of Fas by FasL induces apoptosis by a mechanism that cannot be blocked by Bcl-2 or Bcl-x(L). Proc. Natl. Acad. Sci. USA 96: 14871 – 14876
41. Fulda S, Scaffidi C, Susin SA, Krammer PH, Kroemer G, Peter ME and Debatin KM (1998) Activation of mitochondria and release of mitochondrial apoptogenic factors by betulinic acid. J. Biol. Chem. 273: 33942 – 33948
42. Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang HG, Reed JC, Nicolson DW, Alnemri ES, Green DR and Martin SJ (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
43. Friedrich K, Wieder T, Haefen CV, Radetzki S, Jänicke R, Schulze-Osthoff K, Dorken B and Daniel PT (2001) Overexpression of caspase-3 restores sensitivity for drug-induced apoptosis in breast cancer cell lines with acquired drug resistance. Oncogene 20: 2749 – 2760
44. Jain N, Lam YM, Pym J and Campling BG (1996) Mechanisms of resistance of human small cell lung cancer lines selected in VP-16 and cisplatin. Cancer 77: 1797 – 1808
45. Cutts SM, Parsons PG, Sturm RA and Phillips DR (1996) Adriamycin-induced DNA adducts inhibit the DNA interactions of transcription factors and RNA polymerase. J. Biol. Chem. 271: 5422 – 5429
46. Blagosklonny MV and Fojo T (1999) Molecular effects of paclitaxel: myths and reality (a critical review). Int. J. Cancer 83: 151 – 156
47. Pirnia F, Breuleux M, Schneider E, Hochmeister M, Bates SE, Marti A, Hotz MA, Betticher DC and Borner MM (2000) Uncertain identity of doxorubicin-resistant MCF-7 cells lines expressing mutated p53. J. Natl. Cancer Inst. 92: 1535 – 1536
48. Fritsche M, Haessler C and Brandner G (1993) Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. Oncogene 8: 307 – 318
49. Tishler RB, Calderwood SK, Coleman CN and Price BD (1993) Increases in sequence specific DNA binding by p53 following treatment with chemotherapeutic and DNA damaging agents. Cancer Res. 53: 2212 – 2216
50. Moroni M, Hickman E, Denchi E, Caprara G, Colli E, Cecconi F, Muller H and Helin K (2001) Apaf-1 is a transcriptional target for E2F and p53. Nature Cell Biology 3: 552 – 560
51. Keyomarsi K, Sandoval L, Band V and Pardee AB (1991) Synchronization of tumor and normal cells from G1 to multiple cell cycles by lovastatin. Cancer Res. 51: 3602 – 3609
52. Tsujimoto Y (1989) Overexpression of the human BCL-2 gene product results in growth enhancement of Epstein-Barr virus-immortalized B cells. Proc. Natl. Acad. Sci. USA 86: 1958 – 1962
53. Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS and Dixit VM (1995) Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell 81: 801 – 809
54. Deveraux QL, Leo E, Stennicke HR, Welsh K, Salvesen GS and Reed JC (1999) Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. EMBO J. 18: 5242 – 5251
55. Chinnaiyan AM, O'Rourke K, Tewari M and Dixit VM (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 81: 505 – 512