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Sensitization for death receptor- or drug-induced apoptosis by re-expression of caspase-8 through demethylation or gene transfer

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Resistance of tumors to treatment with cytotoxic drugs, irradiation or immunotherapy may be due to disrupted apoptosis programs. Here, we report in a variety of different tumor cells including Ewing tumor, neuroblastoma, malignant brain tumors and melanoma that caspase-8 expression acts as a key determinant of sensitivity for apoptosis induced by death-inducing ligands or cytotoxic drugs. In tumor cell lines resistant to TRAIL, anti-CD95 or TNFα, caspase-8 protein and mRNA expression was decreased or absent without caspase-8 gene loss. Methylation-specific PCR revealed hypermethylation of caspase-8 regulatory sequences in cells with impaired caspase-8 expression. Treatment with the demethylation agent 5-Aza-2′-deoxycytidine (5-dAzaC) reversed hypermethylation of caspase-8 resulting in restoration of caspase-8 expression and recruitment and activation of caspase-8 at the CD95 DISC upon receptor cross-linking thereby sensitizing for death receptor-, and importantly, also for drug-induced apoptosis. Inhibition of caspase-8 activity also inhibited apoptosis sensitization by 5-dAzaC. Similar to demethylation, introduction of caspase-8 by gene transfer sensitized for apoptosis induction. Hypermethylation of caspase-8 was linked to reduced caspase-8 expression in different tumor cell lines *in vitro* and, most importantly, also in primary tumor samples. Thus, these findings indicate that re-expression of caspase-8, e.g. by demethylation or caspase-8 gene transfer, might be an effective strategy to restore sensitivity for chemotherapy- or death receptor-induced apoptosis in various tumors *in vivo*. *Oncogene* (2001) **20,** 5865–5877.

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Introduction

Cell death by apoptosis plays a pivotal role in the regulation of various physiological or pathological conditions and has also been implied to mediate therapy-induced cytotoxicity, e.g. in response to cytotoxic drug treatment or γ-irradiation (Peter and Krammer, 1998; Griffith and Lynch, 1998; Kaufmann and Earnshaw, 2000; Debatin, 1997; Friesen *et al*., 1996; Fulda *et al*., 1997, 1998a,b). Apoptosis pathways may be initiated through different entry sites, such as death receptors (receptor pathway) or mitochondria (mitochondrial pathway) resulting in activation of effector caspases (Peter and Krammer, 1998; Griffith and Lynch, 1998; Kaufmann and Earnshaw, 2000; Debatin, 1997). Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) results in receptor aggregation and recruitment of the adaptor molecule Fas-associated death domain (FADD) and caspase-8 (Trauth *et al*., 1989; Oehm *et al*., 1992; Kischkel *et al*., 1995; Chinnaiyan *et al*., 1996; Muzio *et al*., 1996; Medema *et al*., 1997; Scaffidi *et al*., 1998). Upon recruitment caspase-8 becomes activated and initiates apoptosis by direct cleavage of downstream effector caspases (Trauth *et al*., 1989; Oehm *et al*., 1992; Kischkel *et al*., 1995; Chinnaiyan *et al*., 1996; Muzio *et al*., 1996; Medema *et al*., 1997; Scaffidi *et al*., 1998; Thornberry and Lazebnik, 1998). A second pathway is initiated at the mitochondrial level (Kroemer and Reed, 2000). Apoptogenic factors such as cytochrome *c*, apoptosis inducing factor (AIF), caspase-2 or caspase-9 are released from mitochondria into the cytosol triggering caspase-3 activation through formation of the cytochrome *c*/Apaf-1/caspase-9-containing apoptosome complex (Kroemer and Reed, 2000; Susin *et al*., 1999a,b; Zou *et al*., 1999). In these cells, caspase-8 is activated downstream of mitochondria promoting further cleavage of effector caspases. Signals originating from the CD95 receptor may be linked to mitochondria by Bid, a BH3 domain containing protein of the Bcl-2 family which assumes cytochrome-*c*-releasing activity upon cleavage by caspase-8 thereby initiating a mitochondrial amplification loop (Kroemer and Reed, 2000; Li *et al*., 1998; Yin *et al*.,

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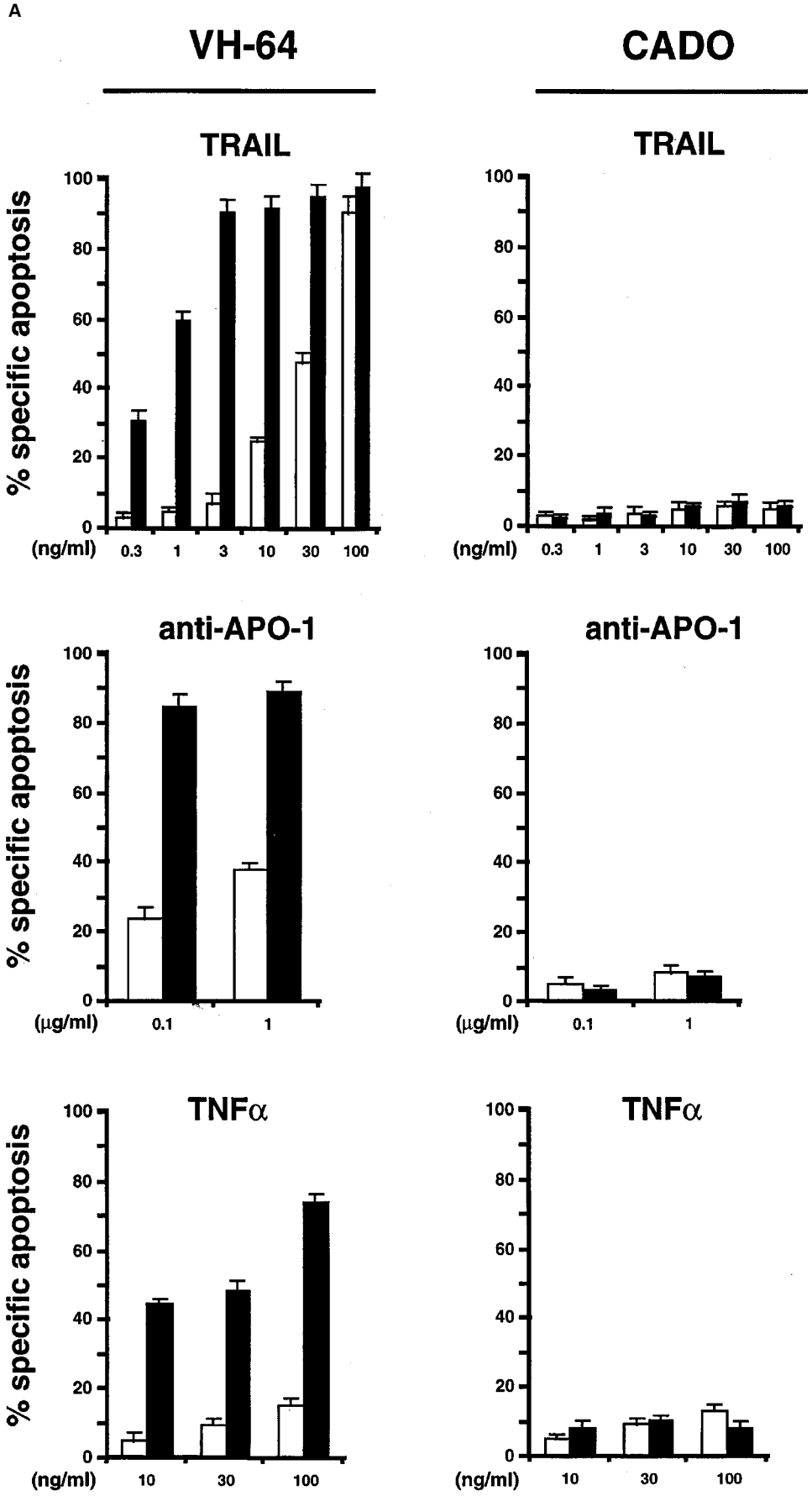
VH-64
CADO
TRAIL
TRAIL
% specific apoptosis
100
80
60
40
20
0
(ng/ml)
0.3
1
3
10
30
100
anti-APO-1
anti-APO-1
% specific apoptosis
(μg/ml)
0.1
1
TNFα
TNFα
% specific apoptosis
(ng/ml)
10
30
100

1999). Studies from caspase-8 knock-out mice indicate that caspase-8 plays a necessary and non-redundant role in various forms of cell death (Varfolomeev *et al*., 1998). Interestingly, mutations in the caspase-8 gene have only infrequently been found in cell lines or primary tumor samples (Teitz *et al*., 2000; Mandruzzato *et al*., 1997).

In addition to genetic alterations, epigenetic modifications such as DNA methylation have recently been implied in loss of gene function and tumorigenesis (Tycko, 2000; Herman and Baylin, 2000). In particular, hypermethylation of normally unmethylated CpG islands located in the promotor region of many tumor suppressor genes or other tumor-related genes, including Rb, p16, p15, VHL, E-cadherin or p73, is associated with loss of expression in cancer cell lines and primary tumors (Tycko, 2000; Herman and Baylin, 2000). Recently, caspase-8 was reported to be silenced by hypermethylation in neuroblastoma with MycN amplification and in peripheral neuroectodermal tumor (PNET) cell lines implying that caspase-8 may act as a tumor suppressor in some tumors (Teitz *et al*., 2000; Hopkins-Donaldson *et al*., 2000; Grotzer *et al*., 2000).

Despite aggressive therapies, resistance of many tumors, e.g. neuroblastoma, malignant brain tumors or soft tissue sarcoma, to current treatment protocols still remains a major concern in cancer therapy (De Vita, 1990). Deregulated apoptosis programs may contribute to tumor progression and treatment resistance. Defects in apoptosis pathways may occur at different levels, at the receptor level, during signal transduction or during the effector phase (Debatin, 1997; Friesen *et al*., 1997; Tschopp *et al*., 1998; Kroemer, 1997; Deveraux and Reed, 1999; Los *et al*., 1997). We previously reported that increased recruitment and activation of caspase-8 through down-regulation of FLIP by metabolic inhibitors may sensitize for CD95-induced apoptosis (Fulda *et al*., 2000). Recently, we found that TNF-related apoptosis-inducing ligand (TRAIL) efficiently triggered apoptosis in the majority of Ewing tumor cell lines including

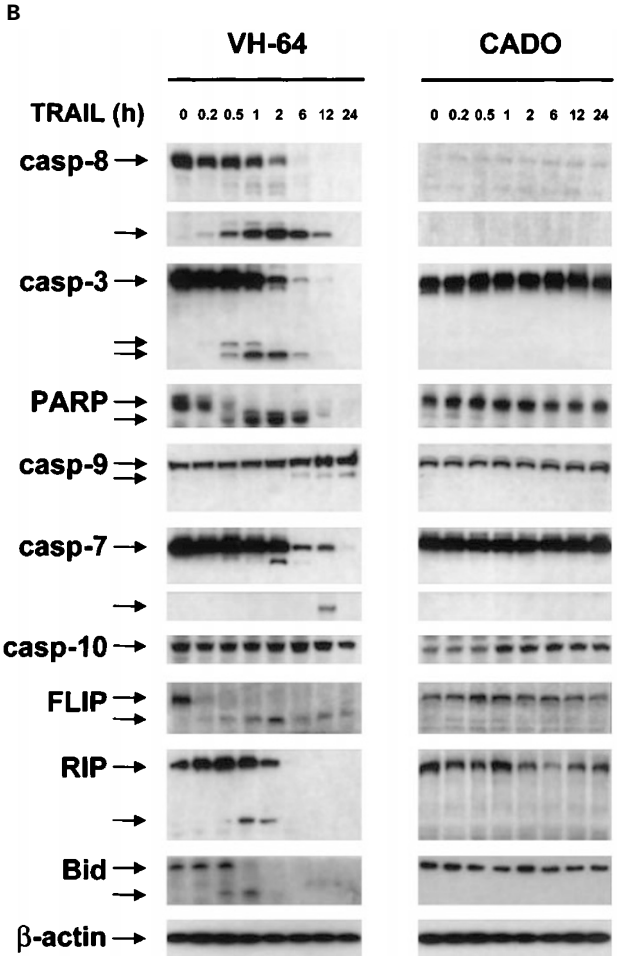

Figure 1 Death receptor-induced apoptosis in sensitive and resistant Ewing tumor cell lines. (**a**) Death receptor-induced apoptosis. Cells were treated for 24 h with 0.3–100 ng/ml TRAIL, 0.1–1 μg/ml anti-APO-1 monoclonal antibody or 10–100 ng/ml TNFα in the absence (□) or presence (■) of 1 μg/ml CHX. Apoptosis was determined by FACS analysis of propidium iodide stained DNA content. (**b**) Activation of apoptosis pathways. Cells were treated for indicated times with 100 ng/ml TRAIL. Cell lysates (50 μg protein per lane) were separated by 12% SDS–PAGE and analysed for protein expression by Western blot. Expression of β-actin was used to control for equal gel loading. Migration positions of cleavage products are indicated by arrows

primary tumor cells, however, a subset of Ewing tumor cells remained resistant even in the presence of protein synthesis inhibitors (van Valen *et al*., 2000). Therefore, we further investigated molecular mechanisms regulating sensitivity or resistance in the present study.

Results

We previously found that TRAIL efficiently triggered apoptosis in the majority of Ewing tumor cell lines (78%, 32/41) and also in primary tumor cells *ex vivo* (100%, 3/3) (van Valen *et al*., 2000). However, a subset of Ewing tumor cell lines remained resistant to TRAIL-induced apoptosis (22%, 9/41) even in the presence of protein synthesis inhibitors (Fulda *et al*., 2000; van Valen *et al*., 2000). Therefore, we further investigated the molecular mechanism(s) regulating sensitivity in these cells.

Death receptor-induced apoptosis in sensitive and resistant Ewing tumor cell lines

Ligation of death receptors by TRAIL, anti-APO-1 (CD95) or TNFα efficiently triggered apoptosis in VH-64 cells, a prototype sensitive Ewing tumor cell line. Caspase-8, -3 and PARP were cleaved after treatment with 100 ng/ml TRAIL for 0.5 h followed by cleavage of caspase-9, -7, FLIP, RIP and Bid without detectable caspase-10 cleavage indicating that apoptosis was predominantly mediated by activation of caspase-8 and -3 (Figure 1a,b). In contrast, no activation of the caspase cascade was found in CADO cells, a prototype Ewing tumor cell line resistant to TRAIL, anti-CD95 of TNFα-induced apoptosis even in the presence of the protein synthesis inhibitor CHX (Figure 1a,b). For further analysis we used VH-64, TC-71 and WE-68 cells as prototype sensitive Ewing tumor cell lines and CADO, GG-62, RDES, SKES, 6647, ES-3 and ET2.1 cells as prototype resistant Ewing tumor cell lines (van Valen *et al*., 2000). ES-2 cells were intermediate sensitive for TRAIL, anti-CD95 or TNFα-induced apoptosis in the presence of CHX.

Expression of death receptors and pro- or antiapoptotic proteins in sensitive and resistant Ewing tumor cell lines

To see whether sensitivity was determined by the presence or absence of pro- or antiapoptotic molecules, we investigated several key molecules of the death receptor pathway (TRAIL receptors 1–4, CD95, TNF-RI, FADD, FLIP, RIP) or the mitochondrial pathway (Bax, Bcl-2, Bcl-X_L, Bcl-X_S, Apaf-1) in addition to IAP's and caspases (caspase-8, -10, -9, -7, -3). Sensitivity for death receptor-induced apoptosis did not correlate with expression of agonistic or antagonistic TRAIL receptors, CD95 or TNF-RI (Figure 2a) or with expression levels of various pro- or antiapoptotic molecules known to regulate apoptosis sensitivity (Figure 2b). However, absent or low expression of caspase-8 was found in resistant Ewing tumor cell lines (CADO, GG-62, RDES, SKES, 6647, ES-3 and ET2.1) compared to sensitive or intermediate sensitive Ewing tumor cell lines (VH-64, TC-71, WE-68, ES-2), whereas expression levels of caspase-10, -9, -7 or -3 protein did not correlate with sensitivity or resistance (Figure 2c).

Impaired caspase-8 expression without loss of the caspase-8 gene

To investigate whether caspase-8 protein expression was downregulated at the transcriptional level, we analysed caspase-8 mRNA expression by RT–PCR. Caspase-8 mRNA expression correlated with protein expression with undetectable levels in cell lines with absent caspase-8 protein expression (CADO, 6647), reduced levels in cell lines with low caspase-8 protein expression (GG-62, RDES, SKES, ET2.1) and high expression in cells with high caspase-8 protein expression (VH-64, ES-2) (Figure 3a,b). However, reduced caspase-8 protein expression was not uniformly associated with reduced caspase-8 mRNA expression, e.g. in ES-3 cells (Figure 3a,b). Despite absent caspase-8 mRNA and protein expression in some cell lines, an intact caspase-8 gene was detected by Southern blot analysis in all cell lines (Figure 3c). The different fragments following digestion of DNA with *Eco*RI represent polymorphisms of the caspase-8 gene generating bands of 8 kb and 4.8 kb (Grenet *et al*., 1999). These findings demonstrate that reduced caspase-8 expression was not the result of gene loss and might be due to reduced transcription or reduced mRNA stability.

Re-expression of caspase-8 by demethylation with 5-dAzaC

Since epigenetic changes such as DNA methylation may result in gene inactivation in the absence of genetic alterations, we tested the effect of the demethylating agent 5-dAzaC on caspase-8 expression which has been reported to promote demethylation of CpG dinucleotides (Jones, 1985). Treatment with 5-dAzaC resulted in a time- and concentration-dependent re-expression of caspase-8 mRNA and protein (Figure 4a). Similarly, upregulation of caspase-8 expression after treatment with 5-dAzaC was also found in several Ewing tumor cell lines with reduced caspase-8 expression, whereas no further increase in caspase-8 levels by treatment with 5-dAzaC was seen in VH-64 or ES-2 cells with already high caspase-8 expression (Figure 4b). These results suggested that reduced caspase-8 expression was the result of caspase-8 hypermethylation.

Impaired caspase-8 expression by hypermethylation

To analyse the DNA methylation status of caspase-8, we performed methylation-specific PCR using primers corresponding to a CpG-rich part of the 5′ flanking

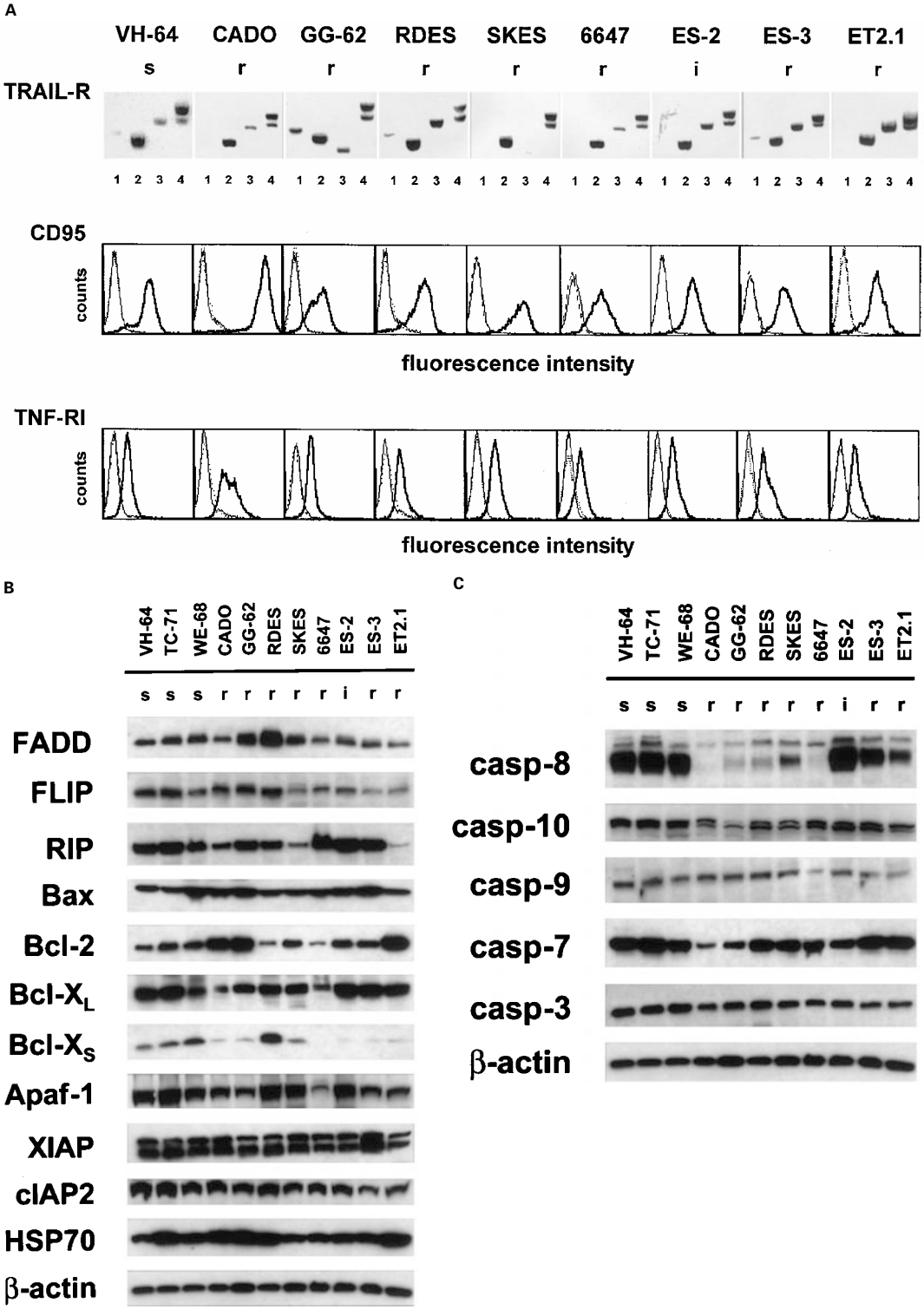

Figure 2 Expression of death receptors and pro- or antiapoptotic proteins in sensitive and resistant Ewing tumor cell lines. (**a**) Expression of death receptors. Expression of TRAIL receptors [DcR1 (lane 1), DcR2 (lane 2), DR4 (lane 3), DR5 (lane 4)] was determined by RT–PCR (upper panel). Expression of β-actin was used to control for RNA integrity and equal gel loading (data not shown). Specificity of PCR-reaction products was controlled by DNA sequencing and the lower migrating band ($\sim$350 bp) of DR4 in GG-62 and SKES cells was identified by DNA sequencing as seryl-tRNA synthetase (data not shown). Expression of CD95 (middle panel) or TNF-RI (lower panel) was determined by flow cytometry using mouse anti-APO-1 monoclonal antibody or mouse anti-CD120a monoclonal antibody followed by phycoerythrin-conjugated anti-mouse IgG antibody. Solid line, cells stained with anti-APO-1 or anti-CD120a antibody; dotted line, cells stained with isotype-matched control antibody; thin line, unstained cells. (**b**) and (**c**) Expression of pro- or antiapoptotic proteins. Cell lysates (50 μg protein per lane) were separated by 12% SDS–PAGE and analysed by Western blot for protein expression

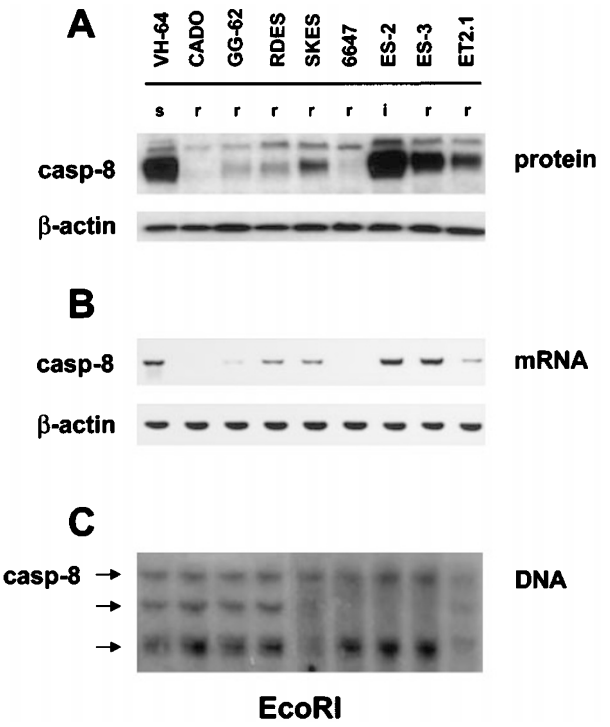

Figure 3 Impaired caspase-8 expression without loss of the caspase-8 gene. Expression of caspase-8 protein was analysed by Western blot (**a**), expression of caspase-8 mRNA by RT–PCR (**b**) or expression of caspase-8 gene by Southern blot (**c**) in Ewing tumor cells. The different polymorphic fragments of caspase-8 following restriction endonuclease digestion of genomic DNA with *Eco*RI that hybridized with the caspase-8 cDNA probe (~11 kb, 8.8 kb, 4.7 kb) are indicated by arrows (Grenet *et al*., 1999)

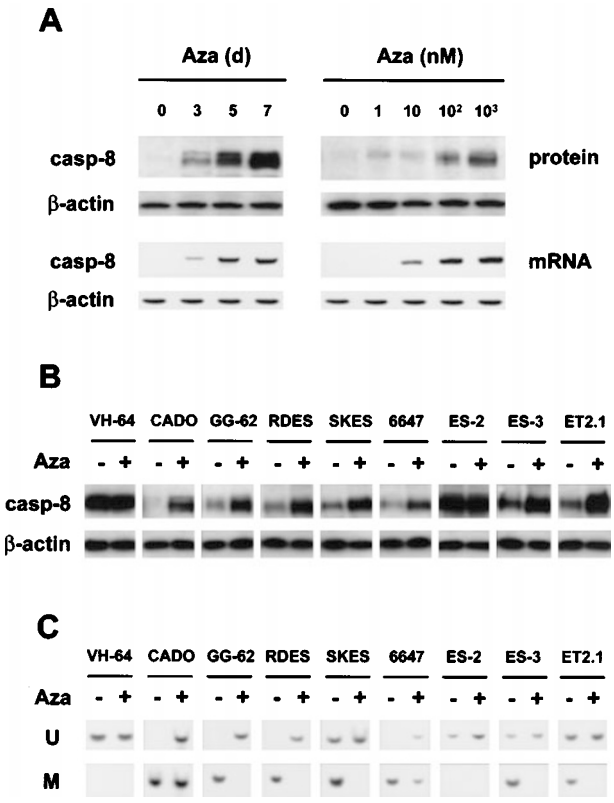

Figure 4 Impaired caspase-8 expression by hypermethylation in Ewing tumor cell lines. (**a**) Time- and concentration-dependent re-expression of caspase-8 by demethylation. CADO Ewing tumor cells were treated with 1 μM 5-dAzaC for 0–7 days (left panel) or with 0–1 μM 5-dAzaC for 5 days (right panel). Expression of caspase-8 protein was analysed by Western blot and expression of caspase-8 mRNA by RT–PCR. (**b**) Re-expression of caspase-8 by demethylation in Ewing tumor cell lines. Cells were left untreated (−) or were treated with 1 μM 5-dAzaC for 5 days (+) and analysed for expression of caspase-8 protein by Western blot. (**c**) Methylation status of caspase-8 in Ewing tumor cell lines. Methylation status of caspase-8 was determined by methylation-specific PCR of cells treated with (+) or without (−) 1 μM 5-dAzaC for 3 days using primers detecting unmethylated (U) or methylated (M) caspase-8 DNA

region of caspase-8 (Teitz *et al*., 2000). The methylation status of caspase-8 regulatory sequences correlated with caspase-8 expression (Figure 4c). In cell lines with high levels of caspase-8 such as VH-64 cells, only the unmethylated form of caspase-8 was found, whereas in cell lines without detectable caspase-8 expression, e.g. CADO cells, caspase-8 was detected only in the methylated form (Figure 4c). Treatment with 5-dAzaC resulted in demethylation of caspase-8 in cell lines with hypermethylated caspase-8 (Figure 4c). Thus, caspase-8 regulatory sequences were hypermethylated in cell lines with reduced caspase-8 expression which was reversed by treatment with the demethylating agent 5-dAzaC.

Impaired caspase-8 expression by hypermethylation in various tumor cell lines and in primary tumor samples

We next analysed different cell lines which displayed low or absent sensitivity for death receptor-induced apoptosis in initial experiments (Fulda *et al*., 2000; and data not shown). Similar to Ewing tumor cell lines, low or absent caspase-8 expression was found in cell lines derived from neuroblastoma, malignant brain tumors, rhabdomyosarcoma or melanoma (Figure 5a), whereas high expression of caspase-8 was detected in various leukemia and lymphoma cell lines which are highly sensitive to death receptor- or drug-induced apoptosis (Figure 5a). Treatment with 5-dAzaC resulted in upregulation of caspase-8 levels in cell lines with reduced expression indicating that caspase-8 expression was impaired by hypermethylation in different tumor cell lines (Figure 5b).

To exclude that our observations were restricted to cell lines maintained in long-term culture, we examined primary tumor samples derived from patients with Ewing tumor, neuroblastoma, medulloblastoma or rhabdomyosarcoma. Methylation-specific PCR revealed that caspase-8 was expressed predominantly in the hypermethylated form in 13 of 20 Ewing tumors, 2 of 3 neuroblastoma, 4 of 10 medulloblastoma and 2 of 4 rhabdomyosarcoma (Figure 5c and Table 1). To see whether hypermethylation of caspase-8 was associated with reduced caspase-8 expression in tumor samples, we determined caspase-8 protein expression by immunohistochemistry. Caspase-8 protein was expressed

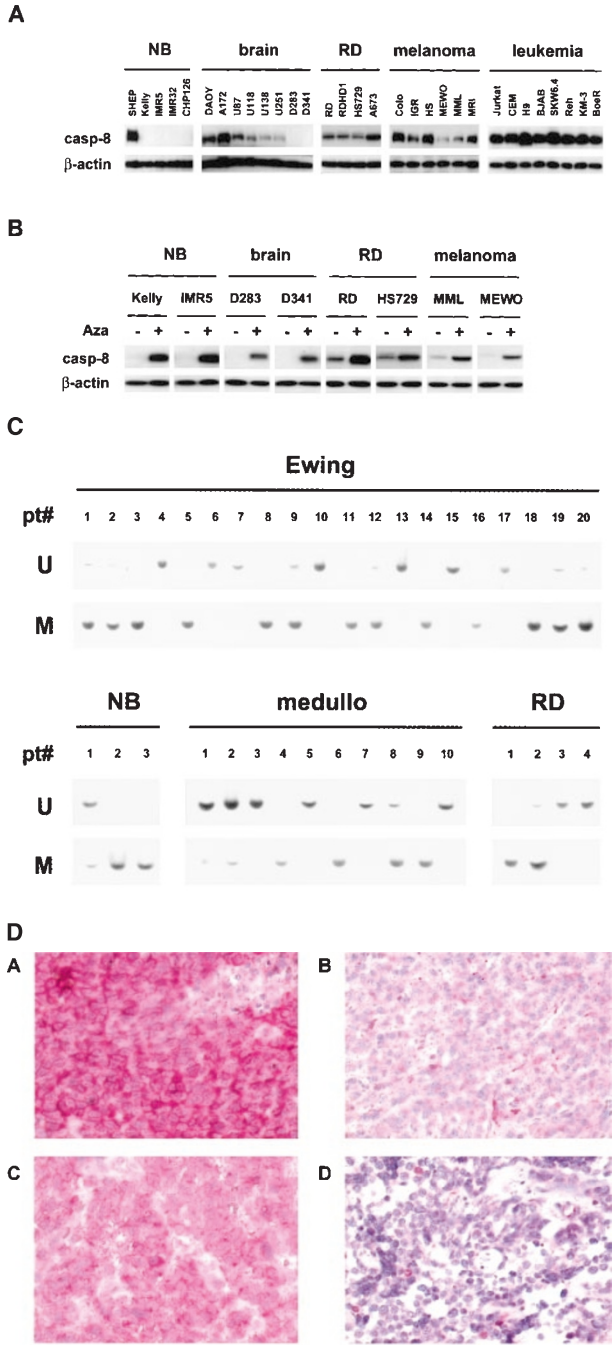

Figure 5 Impaired caspase-8 expression by hypermethylation in neuroectodermal tumor cell lines and tumor samples. (**a**) Caspase-8 expression in neuroectodermal tumor cell lines. Caspase-8 protein expression was analysed in neuroblastoma (NB), malignant brain tumors (brain), rhabdomyosarcoma (RD), melanoma or leukemia cell lines by Western blot. (**b**) Re-expression of caspase-8 by demethylation. Neuroblastoma (NB), malignant brain tumor (brain), rhabdomyosarcoma (RD) or melanoma cell lines were left untreated (−) or were treated with 1 μM 5-dAzaC for 5 days (+) and analysed for expression of caspase-8 protein by Western blot. (**c**) Methylation status of caspase-8 in neuroectodermal tumor samples. Methylation status of caspase-8 was analysed in primary tumor samples derived from patients with Ewing tumor, neuroblastoma (NB), medulloblastoma (medullo) or rhabdomyosarcoma (RD) by methylation-specific PCR as described above using primers detecting unmethylated (U) or methylated (M) caspase-8 DNA. pt#, patient number according to Table 1. (**d**) Caspase-8 expression in neuroectodermal tumor samples. Caspase-8 protein expression in tumor samples was determined by immunohistochemistry (A: Ewing tumor pt#10, B: Ewing tumor pt#12, C: neuroblastoma pt#1, D: rhabdomyosarcoma pt#1; pt#, patient number according to Table 1; magnification $\times 400$)

in tumors with predominantly unmethylated caspase-8, while absent or weak caspase-8 protein expression was found in samples in which caspase-8 was predominantly hypermethylated (Figure 5c,d; Table 1). These results indicate that hypermethylation of caspase-8 regulatory sequences was associated with reduced caspase-8 expression in tumors *in vivo*.

Sensitization for death receptor- or drug-induced apoptosis through re-expression of caspase-8 by demethylation or caspase-8 gene transfer

We then asked whether re-expression of caspase-8 would increase apoptosis sensitivity. Pretreatment

Table 1 Analysis of patient tumor samples

Pt#	*Genetics*	*MSP/u*	*MSP/M*	*ICH*
Ewing sarcoma:				
1. 4640	EWS8/FL17	−/+	+	n.d.
2. 7541	type I. 7/6	−/+	+	n.d.
3. 3439	type II, 7/5	−	+	−
4. 5747	type II, 7/5	+	−	n.d.
5. 16039	t(21;22)	−	+	n.d.
6. 19878	type II, 7/5	+	−	n.d.
7. 10889	t(21;22)	+	−	n.d.
8. 11270	type I, 7/6	−	+	n.d.
9. 17715	type I, 7/6	+	+	+ +
10. 8209	type I, 7/6	+	−	+ + +
11. 13710	type V, 10/5	−	+	n.d.
12. 14095	type VI, 7/8	−/+	+	+
13. 22391	type II, 7/5	+	−	n.d.
14. 36254	type I, 7/6	−	+	n.d.
15. 82	type II, 7/5	+	−	+ +
16. 40360	type I, 7/6	−	+	n.d.
17. 41479	type II, 7/5	+	−	n.d.
18. 8849	type I, 7/6	−	+	n.d.
19. 9775	type V, 10/5	−/+	+	+
20. 17896	type I, 7/6	−/+	+	n.d.
Neuroblastoma:				
1. 3865	MycN 1x	+	+	+ +
2. 8664	MycN 1x	−	+	+
3. 8660	MycN 1x	−	+	+
Medulloblastoma:				
1. D754	n.d.	+	−/+	n.d.
2. D755	n.d.	+	−/+	n.d.
3. D262	n.d.	+	−	n.d.
4. D280	n.d.	−	+	n.d.
5. D398	n.d.	+	−	n.d.
6. D429	n.d.	−	+	n.d.
7. D444	n.d.	+	−	n.d.
8. D446	n.d.	−/+	+	n.d.
9. D447	n.d.	−	+	n.d.
10. D471	n.d.	+	−	n.d.
Rhabdomyosarcoma:				
1. 34697	t(2;13)	−	+	−
2. 17704	t(2;13)	−/+	+	−
3. 34035	t(1;13)	+	−	n.d.
4. 5584	t(1;13)	+	−	n.d.

with 5-dAzaC sensitized different cell lines with low or absent caspase-8 expression for subsequent treatment with TRAIL, anti-APO-1 monoclonal antibody or TNFα (Figure 6a). Since caspase-8 has also been implicated in drug-induced apoptosis, we tested the effect of demethylation on apoptosis following cytotoxic drug treatment. Pretreatment with 5-dAzaC similarly sensitized for apoptosis in response to treatment with doxorubicin, etoposide or cisplatinum (Figure 6a). No further increase in apoptosis sensitivity upon pretreatment with 5-dAzaC was found in VH-64 cells with already high caspase-8 expression (data not shown).

Since treatment with demethylation agents such as 5-dAzaC may modulate expression of several different genes, we further investigated the specific contribution of caspase-8 re-expression for 5-dAzaC-mediated sensitization. First, we analysed caspase-8 function following re-expression of caspase-8. In cell lines treated with 5-dAzaC, re-expressed caspase-8 was recruited to the CD95 death-inducing signaling complex (DISC) upon CD95 triggering or doxorubicin treatment, whereas no recruitment of caspase-8 was found without 5-dAzaC pretreatment (Figure 6b). In contrast, FADD was similarly recruited to CD95 upon stimulation regardless of 5-dAzaC pretreatment (Figure 6b). Similar amounts of CD95 or FADD were found in cellular lysates in the presence or absence of 5-dAzaC (data not shown). Restoration of caspase-8 expression by treatment with 5-dAzaC resulted in cleavage of caspase-8 upon addition of TRAIL, anti-CD95, TNFα or doxorubicin (Figure 6c). Moreover, sensitization for TRAIL or doxorubicin-induced apoptosis following treatment with 5-dAzaC was inhibited in the presence of the caspase-8 specific inhibitor zIETD.fmk or the broad range caspase inhibitor zVAD.fmk (Figure 6d). A dominant negative mutant of caspase-8 also attenuated sensitization for TRAIL and, although to a less extent, for doxorubicin-induced apoptosis (Figure 6d). Furthermore, selective downregulation of FLIP expression by FLIP antisense oligonucleotides synergized with 5-dAzaC to sensitize cells for TRAIL- or doxorubicin-induced apoptosis compared to demethylation treatment alone (Figure 6d) indicating that sensitization by 5-dAzaC involved alterations at the level of the CD95 DISC, e.g. increase in caspase-8 levels. Finally, we stably transfected CADO Ewing tumor cells with a vector containing caspase-8. Caspase-8 expression in single cell clones was confirmed by Western blot (Figure 7a). Cells expressing caspase-8 were significantly more sensitive to treatment with TRAIL or doxorubicin compared to the parental cell line or cells containing control vector (Figure 7b). Thus, by demonstrating that restoration of caspase-8 expression and function by demethylation or by caspase-8 gene transfer sensitized for apoptosis induction, our findings strongly indicate that caspase-8 is a crucial regulator of sensitivity for death receptor- or chemotherapy-induced apoptosis.

Discussion

Since resistance to anticancer therapy may be caused by defective apoptosis programs, the identification of molecular defect(s) in apoptosis signaling may be pivotal to design novel strategies that specifically target resistance. Here, we report in various tumor cells that caspase-8 is a key determinant of sensitivity for apoptosis induced by death-inducing ligands or cytotoxic drugs. Caspase-8 expression was frequently impaired by hypermethylation in different tumor cells, e.g. Ewing tumor, neuroblastoma, malignant brain tumors, rhabdomyosarcoma or melanoma cells both *in vitro* and *in vivo*, and re-expression of caspase-8 by demethylation or caspase-8 gene transfer sensitized for death-receptor- or drug-induced apoptosis. This conclusion is based on a number of independent pieces of evidence:

First, absent or reduced caspase-8 expression was the result of caspase-8 inactivation by DNA hypermethylation without loss or large genomic alterations of the caspase-8 gene. The methylation status of caspase-8 regulatory sequences correlated with caspase-8 expression in tumor cell lines and, most importantly, also in primary tumor samples. Treatment with the demethylating agent 5-dAzaC reversed hypermethylation of caspase-8 resulting in a time- and concentration-dependent re-expression of caspase-8 mRNA and protein.

Second, sensitivity to death receptor-induced apoptosis correlated with caspase-8 expression. Caspase-8 expression was decreased or absent in neuroectodermal tumor cell lines resistant to TRAIL, anti-CD95 or TNFα. Restoration of caspase-8 expression by demethylation resulted in restoration of caspase-8 function, e.g. recruitment and activation of caspase-8 at the CD95 DISC upon receptor cross-linking sensitizing cells with reduced caspase-8 expression for death-receptor- or drug-induced apoptosis. Sensitization was crucially mediated through activation of caspase-8, since inhibition of caspase-8 activity by a caspase-8 specific inhibitor also inhibited apoptosis sensitization by demethylation. Restoration of caspase-8 protein expression by caspase-8 gene transfer similarly sensitized for apoptosis induction. Furthermore, selective downregulation of FLIP by FLIP antisense oligonucleotides synergized with 5-dAzaC to sensitize cells with absent caspase-8 expression for death receptor triggering or drug treatment. However, the effect of 5-dAzaC on expression of other apoptosis-modulating genes in addition to caspase-8 or on other cellular functions, e.g. cell cycle (Jones, 1985), may also contribute to its sensitizing effect. While we found no effect on expression levels of other caspases including caspase-3, -7, -9 or -10, proapoptotic proteins of the Bcl-2 family or signaling molecules of the death receptor pathway such as FADD, treatment with 5-dAzaC resulted in increased expression of DR4 (data not shown). However, upregulation of DR4 by demethylation may not confer sensitivity to apoptosis in cells lacking caspase-8, since caspase-10 could not replace

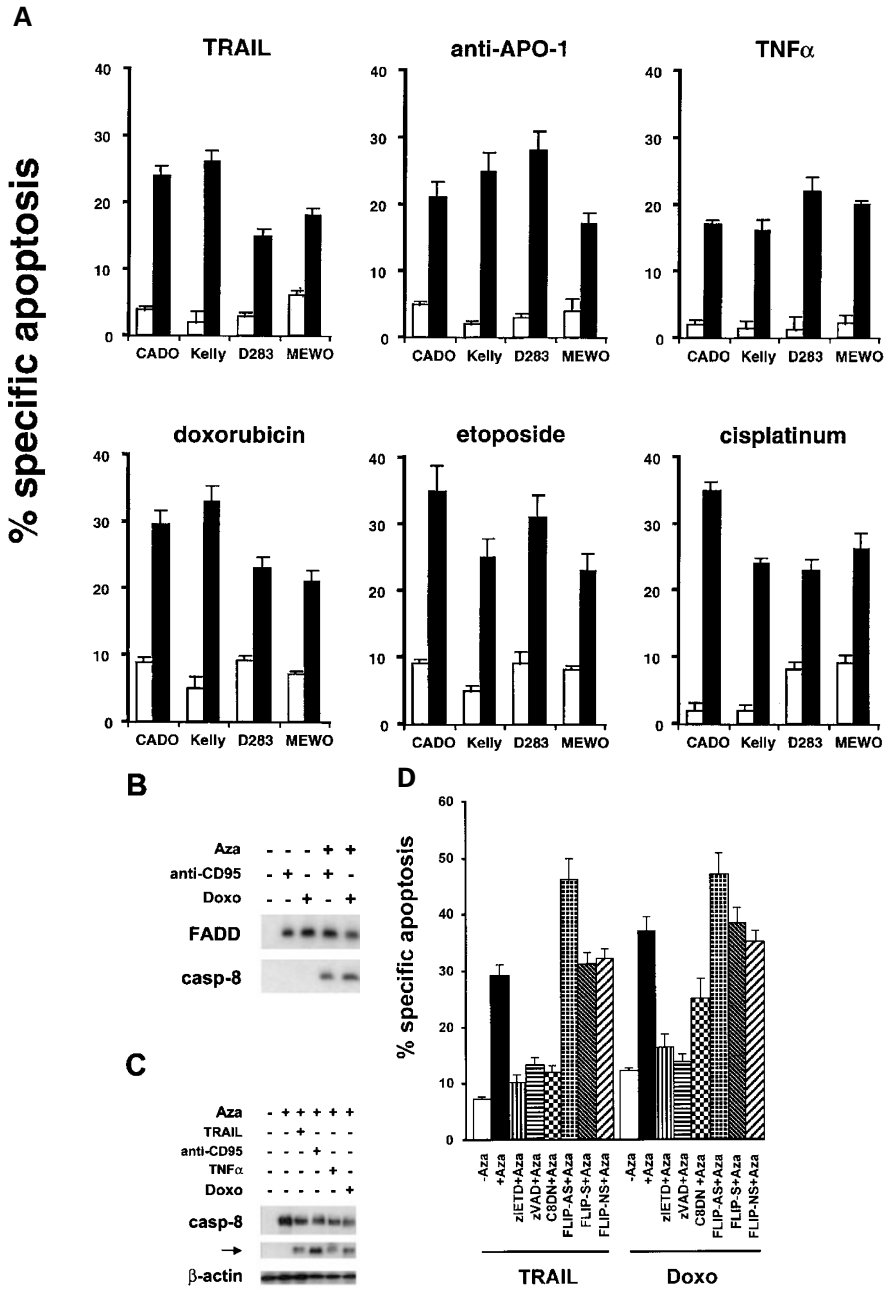

Figure 6 Sensitization for death receptor- or drug-induced apoptosis through re-expression of caspase-8 by demethylation. (**a**) Sensitization for death receptor- or drug-induced apoptosis by demethylation. Cells were left untreated (white bars) or were treated with 10 μM 5-dAzaC for 3 days (black bars) followed by treatment for 24 h with 100 ng/ml TRAIL, 1 μg/ml anti-APO-1 monoclonal antibody or 100 ng/ml TNFα and 1 μg/ml CHX, 0.03 μg/ml doxorubicin, 0.3 μg/ml etoposide or 0.1 μg/ml cisplatinum in the absence (white bars) or presence (black bars) of 10 μM 5-dAzaC. (**b**) Recruitment of re-expressed caspase-8 to the CD95 DISC. CADO Ewing tumor cells were left untreated or were treated with 10 μM 5-dAzaC for 3 days followed by treatment with 2 μg/ml anti-APO-1 (CD95) monoclonal antibody for 2 h or with 0.1 μg/ml doxorubicin for 12 h in the absence or presence of 10 μM 5-dAzaC. Recruitment of FADD or caspase-8 to CD95 was analysed by immunoprecipitation. (**c**) Cleavage of re-expressed caspase-8 upon apoptosis induction. CADO Ewing tumor cells were left untreated or were treated with 10 μM 5-dAzaC for 3 days followed for 24 h by treatment with 100 ng/ml TRAIL, 1 μg/ml anti-APO-1 (CD95) monoclonal antibody, 100 ng/ml TNFα and 1 μg/ml CHX or with 0.1 μg/ml doxorubicin in the absence or presence of 10 μM 5-dAzaC and analysed for caspase-8 cleavage by Western blot. (**d**) Inhibition of 5-dAzaC-mediated sensitization by caspase inhibitors and synergy of 5-dAzaC-mediated sensitization with FLIP antisense oligonucleotides. CADO Ewing tumor cells were left untreated or were treated with 10 μM 5-dAzaC for 3 days followed by treatment for 24 h with 100 ng/ml TRAIL and 1 μg/ml CHX or 0.03 μg/ml doxorubicin in the absence or presence of 10 μM 5-dAzaC, or in the presence of 10 μM 5-dAzaC and 50 μM zIETD.fmk, 50 μM zVAD.fmk, 1 μM FLIP antisense oligonucleotides (FLIP-AS), 1 μM FLIP sense oligonucleotides (FLIP-S) or 1 μM FLIP nonsense oligonucleotides (FLIP-NS). Specific apoptosis of cells treated with 10 μM 5-dAzaC or with 10 μM 5-dAzaC and 50 μM zIETD.fmk, 50 μM zVAD.fmk, 1 μM FLIP antisense oligonucleotides, 1 μM FLIP sense oligonucleotides, 1 μM FLIP nonsense oligonucleotides or dominant negative caspase-8 (C8DN) without TRAIL or doxorubicin was $<4\%$, transfection with an empty control vector had no effect on 5-dAzaC-mediated sensitization (data not shown)

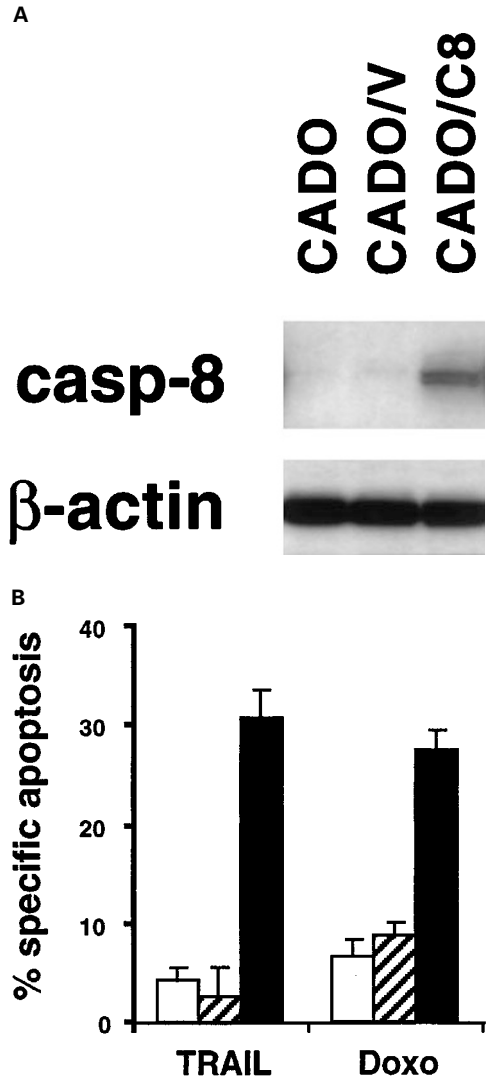

Figure 7 Sensitization for death receptor- or drug-induced apoptosis by caspase-8 gene transfer. (**a**) Expression of caspase-8. Caspase-8 protein expression was analysed by Western blot in CADO Ewing tumor cells or CADO Ewing tumor cells stably transfected with vector control (CADO/V) or caspase-8 cDNA (CADO/C8). (**b**) Sensitization for death receptor- or drug-induced apoptosis by caspase-8 gene transfer. Cells [CADO (white bars), CADO cells transfected with vector control (CADO/V, hatched bars) or caspase-8 cDNA (CADO/C8, black bars)] were treated for 24 h with 100 ng/ml TRAIL and 1 μg/ml CHX or 0.03 μg/ml doxorubicin. Specific apoptosis was determined and calculated as described above. Data are shown for representative clones, similar results were obtained using different clones with high caspase-8 expression or vector control, respectively

the lack of caspase-8 to initiate activation of the caspase cascade in these cells (Figure 1b) and caspase-10 has not been found to be directly involved in the TRAIL or CD95 pathway (Bodmer *et al*., 2000; Sprick *et al*., 2000; Kischkel *et al*., 2000; Juo *et al*., 1998).

In recent years, epigenetic alterations such as hypermethylation of CpG-rich islands within the promotor region have been recognized as an alternative mechanism to genetic changes for loss of gene function in various tumor systems (Herman and Baylin, 2000; Tycko, 2000). At present, the mechanism(s) for tumor-specific abnormal DNA methylation patterns are not exactly known. Overexpression of DNA methyltransferases (DNMT's) has been reported in some tumor cells, although the exact role of DNMT's in deregulated DNA methylation remains unclear (Robertson *et al*., 1999; Hendrich and Bird, 2000). In addition, tumor-specific genetic alterations such as amplification of oncogenes or chromosomal translocations may predispose for abnormal methylation patterns. In the neuroblastoma cell lines analysed in this study, hypermethylation of the caspase-8 gene was only found in cell lines with amplification of the MycN gene consistent with a recent report (Teitz *et al*., 2000). However, caspase-8 levels were similar in neuroblastoma cells with tetracycline-controlled or stable overexpression of MycN (data not shown) indicating that silencing of the caspase-8 gene was not a direct consequence of increased MycN expression. Since MycN gene amplification is often preceded by 1p36 loss of heterozygosity in neuroblastoma tumors (Maris and Matthay, 1999), loss of function of tumor suppressor gene(s) predicted to reside in this region may contribute to the methylation-induced inactivation of caspase-8 in MycN-amplified neuroblastoma. The majority of Ewing tumors are characterized by t(11;22) or t(21;22) chromosomal translocations resulting in EWS/FLI-1 or EWS/ERG fusion genes, while t(1;13) or t(2;13) chromosomal translocations, juxtaposing PAX7 and PAX3, respectively, with the forkhead domain gene FKHR, are frequentially found in rhabdomyosarcoma (Anderson *et al*., 1999; van Valen, 1999). The resulting fusion proteins can function as aberrant transcriptional activators, however, so far they have not been implicated in tumor-specific abnormal methylation patterns (Anderson *et al*., 1999; van Valen, 1999).

Re-expression of caspase-8 restored sensitivity for death receptor-mediated apoptosis, and most importantly, also for drug-induced apoptosis. These findings are consistent with genetic studies using embryonic fibroblasts derived from caspase-8 knock-out mice or mutant Jurkat leukemia cells lacking caspase-8 showing that caspase-8 played an essential and non-redundant role in TRAIL or CD95 signaling (Varfolomeev *et al*., 1998; Juo *et al*., 1998) and also participated in drug-induced apoptosis (Juo *et al*., 1998). An increase in chemosensitivity upon re-expression of caspase-8 from 5–10% to 30–35% may be clinically meaningful, since it may critically affect the time required for execution of the death program (Tang *et al*., 2000). Caspase-8 has been implied to mediate apoptosis upon stimulation of both the death receptor and/or the mitochondrial pathway and may initiate a mitochondrial amplification loop by mediating Bid cleavage (Peter and Krammer, 1998; Fulda *et al*., 1998a; Scaffidi *et al*., 1998; Tang *et al*., 2000). In addition, anticancer agents can initiate apoptosis and activation of caspase-8 through both the death receptor and/or the mitochon-

drial pathway, depending on the cell type (Fulda *et al*., 2001).

Recently, caspase-8 was reported to be silenced by hypermethylation in a subset of neuroblastoma and in PNET (Teitz *et al*., 2000; Hopkins-Donaldson *et al*., 2000; Grotzer et al., 2000). In the present study, we found that caspase-8 is a key determinant of sensitivity for apoptosis induction in a variety of different tumor cells including neuroblastoma, Ewing tumor, rhabdomyosarcoma, malignant brain tumors and melanoma. These findings indicate that impaired caspase-8 expression by hypermethylation might be a more frequent mechanism to evade apoptosis induction than initially thought. Importantly, restoration of caspase-8 expression not only sensitized tumor cell lines for death receptor-triggered apoptosis, but also for drug-induced apoptosis. Since caspase-8 expression was frequently impaired by DNA hypermethylation both *in vitro* and *in vivo*, our findings may have important implications for rational strategies targeting resistance. Our data also indicate that novel concepts using TRAIL in the treatment of tumors would only be effective in tumors in which sufficient levels of caspase-8 are expressed in the tumor cells. Thus, upregulation of caspase-8 expression, e.g. by demethylation or by caspase-8 gene transfer, might be an effective strategy to sensitize various different tumor cells for chemotherapy- or death receptor-induced apoptosis.

Materials and methods

Cell culture and tumor samples

Ewing tumor [VH-64, TC-71, WE-68, CADO-ES-1 (CADO), GG-62, RDES, SKES-1 (SKES), 6647, ES-2, ES-3, STA-ET-2.1 (ET2.1) (van Valen, 1999)], neuroblastoma (SHEP, Kelly, IMR5, IMR32, CHP126), glioblastoma [A172, U87MG (U87), U118MG (U118), U138MG (U138), U251MG (U251)], medulloblastoma [Daoy, D283Med (D283), D341Med (D341)], melanoma [Colo-38 (Colo), IGR, HS-69-ST (HS), MEWO, MML-1 (MML), MRI-H-221 (MRI)], rhabdomyosarcoma (RD, RDHD1, HS729, A673) or leukemia cells (Jurkat, CEM, H9, BJAB, SKW6.4, Reh, KM-3, BoeR) were maintained in RPMI 1640 medium (Life Technologies, Inc., Eggenstein, Germany) as previously described (Fulda *et al*., 1997). 0.5×10^5 cells/ml were cultured in 24-well-plates for determination of apoptosis or in 75 cm^2 flasks (Falcon, Heidelberg, Germany) for protein isolation. Tumor samples from Ewing tumor, neuroblastoma, medulloblastoma or rhabdomyosarcoma were obtained from the European Intergroup Cooperative Ewing's Sarcoma Study (EICESS) group, the German neuroblastoma study group, the German Hirntumor (HIT) study group or the German Cooperative Weichteilsarkom (CWS) study group, respectively.

Determination of apoptosis

Cells were incubated with mouse anti-APO-1 IgG3 monoclonal antibody (Trauth *et al*., 1989), recombinant human TRAIL (PeproTech Inc., Rocky Hill, NJ, USA), TNFα (Calbiochem, Bad Soden, Germany), doxorubicin (Amersham Pharmacia, Freiburg, Germany), cisplatinum (Sigma, Deisenhofen, Germany), etoposide (Bristol Myers, Munich, Germany), benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk; Bachem, Heidelberg, Germany) or benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone (zIETD.fmk; Bachem), cycloheximide (CHX, Sigma) or 5-Aza-2′-deoxycytidine (5-dAzaC; Sigma). Quantification of DNA fragmentation was performed by fluorescence-activated cell-sorting (FACS) analysis of propidium iodide stained nuclei as previously described (Fulda *et al*., 1997; Nicoletti *et al*., 1991). Percentage of specific apoptosis was calculated as follows: $100 \times$[experimental apoptosis (%) – spontaneous apoptosis (%)/100% – spontaneous apoptosis (%)]. Mean and s.d. of triplicates are shown, similar results were obtained in three independent experiments. Sensitivity (s) was defined by $>60\%$ specific apoptosis, intermediate sensitivity (i) by 25–60% specific apoptosis and resistance (r) by $<25\%$ specific apoptosis after treatment for 24 h with 100 ng/ml TRAIL, 1 μg/ml anti-APO-1 monoclonal antibody or 100 ng/ml TNFα and 1 μg/ml CHX.

Western blot analysis and immunoprecipitation

Western blot analysis was performed as previously described (Fulda *et al*., 1997) using mouse anti-caspase-8 monoclonal antibody C15 (1 : 10 dilution of hybridoma supernatant) (Scaffidi *et al*., 1997), mouse anti-caspase-3 monoclonal antibody (1 : 1000, Transduction Laboratories, Lexington, KY, USA), mouse anti-caspase-7 monoclonal antibody (1 : 1000, Transduction Laboratories), rabbit anti-caspase-9 polyclonal antibody (1 : 1000, PharMingen, San Diego, CA, USA), rabbit anti-caspase-10 polyclonal antibody (1 : 1000, PharMingen), rabbit anti-cIAP2 polyclonal antibody (1 : 1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-Bax polyclonal antibody (1 : 1000, Upstate Biotechnology, Lake Placid, NY, USA), rabbit anti-Bid polyclonal antibody (Trevigen, Gaithersburg, MD, USA), mouse anti-Bcl-2 monoclonal antibody (1 : 1000, Santa Cruz Biotechnology), rabbit anti-Bcl-X_L polyclonal antibody (1 : 1000, Santa Cruz Biotechnology), mouse anti-HSP70 monoclonal antibody (1 : 1000, Stressgen Biotechnologies Corp., Victoria, Canada), mouse anti-Apaf-1 monoclonal antibody (1 : 1000, Stressgen), mouse anti-FADD monoclonal antibody (1 : 1000, Transduction Laboratories), mouse anti-FLIP monoclonal antibody (1 : 1000, PharMingen), rabbit anti-poly(ADP-ribose) polymerase (PARP) polyclonal antibody (1 : 5000, Boehringer Mannheim, Mannheim, Germany), mouse anti-receptor-interacting kinase (RIP) monoclonal antibody (1 : 1000, Transduction Laboratories), mouse anti-XIAP monoclonal antibody (1 : 1000, Transduction Laboratories) or mouse anti-β-actin monoclonal antibody (1 : 5000, Sigma) followed by goat anti-mouse IgG or goat anti-rabbit IgG (1 : 5000, Santa Cruz Biotechnology). Enhanced chemiluminescence (ECL, Amersham Pharmacia) was used for detection. Expression of β-actin was used to control for equal gel loading. Immunoprecipitation of the CD95 death-inducing signaling complex (DISC) was performed as previously described (Scaffidi *et al*., 1998).

Analysis of CD95 or TNF-RI expression

Cells were stained with anti-APO-1 (CD95) IgG1 monoclonal antibody [1 μg/ml (Trauth *et al*., 1989)] or anti-CD120a IgG1 monoclonal antibody (1 μg/ml, Caltac Laboratories, Burlingham, CA, USA) as previously described (Fulda *et al*., 1997).

Downregulation of FLIP protein expression by FLIP antisense oligonucleotides

Phosphorothiorate antisense oligodeoxynucleotides to inhibit the FLIP initiation codon, control sense and nonsense oligodeoxynucleotides (Interactiva Biotechnologie GmbH, Ulm, Germany) with the following published sequences (Perlman *et al*., 1999) were used as previously described (Fulda *et al*., 2000): FLIP antisense (FLIP-AS) 5′-GACTTCAGCAGACATCCTAC-3′, FLIP sense (FLIP-S) 5′-CATCCTACAGACGACTTCAG-3′, FLIP nonsense (FLIP-NS) 5′-TGGATCCGAACATGTCAGA-3′. The uptake of FITC-conjugated oligonucleotides was controlled by flow cytometry (Fulda *et al*., 2000).

RT–PCR and methylation-specific PCR

RT–PCR was performed as previously described (Fulda *et al*., 1997). Genomic DNA was isolated using the Qiagen blood and cell culture DNA kit (Qiagen, Hilden, Germany). Methylation-specific PCR was performed as previously described (Herman *et al*., 1996) using primers corresponding to a CpG-rich region of the 5′ flanking region of caspase-8 (Teitz *et al*., 2000). Genomic DNA not treated with bisulfite (unmodified) was not amplified with either the methylated or unmethylated primers, peripheral blood lymphocytes were used as positive control for unmethylated caspase-8 and *in vitro* methylated DNA as positive control for methylated DNA (data not shown). The following primer sequences were used (Interactiva Biotechnologie GmbH): caspase-8 unmethylated-specific 5′-TAGGGGAATTTGGAGATTGTGA-3′ and 5′-CCATATATATCTACTTCAAAACAA-3′, caspase-8 methylated-specific 5′-TAGGGGATTCGGACATTGCGA-3′ and 5′-CGTATATCTACATTCGAAACGA-3′, caspase-8 5′-CAGCATTAGGGACAGGAATC-3′ and 5′-CAGTTATTCACAGTGGCCAT-3′, TRAIL-R1 [death receptor 4 (DR4)] 5′CCTCGGCTCCGGGTCCACAAG′ and 5′-TGAGCCGATGCAACAACAGACAAT-3′, TRAIL-R2 [death receptor 5 (DR5)] 5′-GAGAGCGGCCCCACAACAAAAGA-3′ and 5′-CCTGGGTGGGCTGCAAGATACTCA-3′, TRAIL-R3 [decoy receptor 1 (DcR1)] 5′-CCGGATCCCCAAGACCCTAAA-3′ and 5′-TGGCACCAAATTCTTCAACACA-3′, TRAIL-R4 [decoy receptor 2 (DcR2)] 5′-CCCCCGGCAGGACGAAGTT-3′ and 5′-CTCCTCCGCTGCTGGGGTTTT-3′. Expression of β-actin (Stratagene, Heidelberg, Germany) was used as standard for RNA integrity and equal gel loading. Specificity of PCR-reaction products was controlled by DNA sequencing. PCR-reaction products were run on a 1.5% agarose gel, stained with ethidium bromide and visualized by UV illumination.

Southern blot analysis

Genomic DNA was digested with *Eco*RI (Amersham Pharmacia), resolved by agarose gel electrophoresis and transferred to HybondN+ nitrocellulose membrane (Amersham Pharmacia). Membranes were hybridized with a ^{32}P-γ-dCTP-labeled full-length caspase-8 cDNA fragment (Grenet *et al*., 1999) overnight at 65°C, washed three times with 0.1%SSC/0.1%SDS and visualized by exposure to hyperfilm (Amersham Pharmacia) at −80°C.

Caspase-8 gene transfer

CADO cells were transfected with pcDNA3 vector containing caspase-8 cDNA (Grenet *et al*., 1999), dominant negative caspase-8 (Fernandes-Alnemri *et al*., 1996) or empty vector using effectene transfection reagent (Qiagen) and cultured in 0.5 mg/ml G418 (Life Technologies, Inc.). Cotransfection with pDsRed1-N1 plasmid (Clontech Laboratories) was performed for transient transfection to identify transfected cells (>70% transfection efficacy).

Immunohistochemistry (ICH)

Formalin fixed and paraffin embedded sections of corresponding tumor material were cut at 3–4 μm and mounted on poly-L-lysine coated glass slides. Sections were immersed in 0.01 M sodium citrate buffer, pH 6.0, and antigen retrieval was performed for 10 min in a laboratory autoclave at 120°C. Rabbit anti-caspase-8 polyclonal antibody (1:200; H-134, Santa Cruz Biotechnology) was applied for 45 min at room temperature, followed by mouse-anti-rabbit (RPMI, 1:30; 30 min at room temperature, Dako, Copenhagen, Denmark) and rabbit-anti-mouse phosphatase-anti-alkaline phosphatase (APAAP) complex (RPMI, 1:100; 60 min at room temperature, Dako). Sections were rinsed in distilled water for 10 min, counterstained with hematoxylin and mounted in Kayser's glycerine gelatine. Omission of the primary antibody served as negative control. Caspase-8 protein expression was classified as follows: absence (−), weak expression (+), presence (++), strong expression (+++), not determined (n.d.).

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