Cancer Therapy: Preclinical

Clinical Cancer Research

Important Role of Caspase-8 for Chemosensitivity of ALL Cells

Harald Ehrhardt[1,2], Franziska Wachter[1], Martina Maurer[1], Karsten Stahnke[4], and Irmela Jeremias[1,3]

Abstract

Purpose: Sensitivity of tumor cells toward chemotherapy mainly determines the prognosis of patients suffering from acute lymphoblastic leukemia (ALL); nevertheless, underlying mechanisms regulating chemosensitivity remain poorly understood. Here, we aimed at characterizing the role of caspase-8 for chemosensitivity of B- and T-ALL cells.

Experimental Design: Primary tumor cells from children with ALL were evaluated for expression levels of the caspase-8 protein, were amplified in nonobese diabetic/severe combined immunodeficient mice, transfected with siRNA, and evaluated for their chemosensitivity *in vitro*.

Results: Effective cell death in B- and T-ALL cells depended on the presence of caspase-8 for the majority of cytotoxic drugs routinely used in antileukemia treatment. Caspase-8 was activated independently from extrinsic apoptosis signaling. Accordingly in primary ALL cells, the expression level of caspase-8 protein correlated with cell death sensitivity toward defined cytotoxic drugs *in vitro*. In the subgroup of primary ALL cells, with low expression of caspase-8, methotrexate (MTX) upregulated the expression of caspase-8 mediated by the transcription factor p53, suggesting epigenetic silencing of caspase-8. RNA interference in patient-derived B- and T-ALL cells revealed that effective cell death induction by most routine drug combinations involving MTX depended on the presence of caspase-8.

Conclusion: Our results indicate that caspase-8 is crucial for the high antileukemic efficiency of numerous routine cytotoxic drugs. Reexpression of epigenetically downregulated caspase-8 represents a promising approach to increase efficiency of antileukemic therapy. *Clin Cancer Res; 17(24); 7605–13. ©2011 AACR.*

Introduction

Downregulation of proapoptotic signaling proteins represents an important mechanism, how tumor cells evade anticancer treatment. As an example, caspase-8 is frequently downregulated in tumor cells of different origins, mostly due to epigenetic gene silencing by promoter hypermethylation (1–3). Demethylating agents such as azacythidine induce reexpression of caspase-8 and sensitize for caspase-8–dependent apoptosis (1, 2). Loss or dysfunction of caspase-8 result in increased cellular transformation, enhanced tumor progression, poor response to chemotherapy, and impaired prognosis of patients of different cancer entities (4–8).

Several attempts were undertaken to upregulate the expression of caspase-8 in tumor cells. The caspase-8 promoter contains binding sites for NFκB, SP-1, IRF-1, and p53, and efficient upregulation of caspase-8 was obtained upon stimulation of the STAT-1/IRF-1 pathway (2). For epigenetically downregulated caspase-8, azacythidine and IFN-γ were effective (9, 10). In addition to azacythidine, we have recently shown that epigenetically downregulated caspase-8 can be upregulated by routinely used cytotoxic drugs such as methotrexate (MTX) via the transcription factor p53 (11).

Caspase-8 is mainly known for its important role within the extrinsic apoptosis pathway, where it signals from proximal death receptor activation to activation of caspases or mitochondria (12, 13). In contrast, cytotoxic drugs mainly signal via the intrinsic apoptosis signaling pathway, where activated mitochondria release apoptogenic factors such as cytochrome c for cleavage of downstream effector caspases (14, 15). The role of caspase-8 for intrinsic apoptosis signaling has been controversially discussed (1, 16–19). Recent data provided evidence for an downstream amplifier function of caspase-8 during drug-induced cell

Authors' Affiliations: [1]Helmholtz Center Munich-German Research Center for Environmental Health; [2]Division of Neonatology, University Children's Hospital, Perinatal Center, Ludwig-Maximilians-University Munich, Campus Großhadern; [3]Department of Oncology/Hematology, Dr. von Haunersches Kinderspital, Munich; and [4]University Children's Hospital, Eythstr, Ulm, Germany

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Corresponding Author: Irmela Jeremias, Helmholtz Center Munich–German Research Center for Environmental Health, Department of Gene Vectors, Marchioninistrasse 25, D-81377 Munich, Germany. Phone: 49-89-7099-424; Fax: 49-89-7099-225; E-mail: Irmela.Jeremias@helmholtz-muenchen.de

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Translational Relevance

Our data represent the basis to select targeted drug combinations for future treatment trials of acute lymphoblastic leukemia (ALL). We describe that the proapoptotic intracellular signaling protein caspase-8 is required for antileukemia efficiency of a high number of cytotoxic drugs, including asparaginase, cyclophosphamide, dexamethasone, and doxorubicin. Epigenetic silencing of proapoptotic proteins represents a central regulatory mechanism of how tumor cells escape chemosensitivity, and we found epigenetic silencing of caspase-8 expression in a number of primary ALL samples. Expression of caspase-8 should be evaluated as a potential biomarker for risk stratification in ALL. Methotrexate (MTX) induces reexpression of epigenetically downregulated caspase-8. Therefore, targeted drug combinations can consist of MTX together with drugs depending on the expression of caspase-8. Retrospectively, our data might explain on a molecular level why clinical empirical studies already revealed a high antileukemic efficiency for some of these drug combinations over decades.

death in some but not all tumor types (20, 21). For acute lymphoblastic leukemia (ALL), the role of caspase-8 remains poorly defined. Interestingly, a recent paper showed that in patients with childhood ALL, high mRNA levels of caspase-8 were predictive for a good initial response to polychemotherapy (8).

We aimed to characterize the functional relevance of caspase-8 for cytotoxic drug-induced apoptosis in patient-derived childhood ALL cells. Using molecular studies, we found a surprisingly important function of caspase-8 for chemosensitivity of ALL cells.

Materials and Methods

Materials

TRAIL from Pepro (Peprotech) without his-tag was used exclusively for all experiments. Anti-apo1, Fas receptor blocking antibody, DR4 and DR5 Fc fragments, and zIETD were obtained by Alexis Corp., MTX from Calbiochem, 4-hydroperoxy-cyclophosphamide from Baxter oncology GmbH. All further reagents from Sigma. For Western blot, the following antibodies were used: anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) from Thermo Fisher, anti-p53 and anti-Lamin A/C from Santa Cruz and anti–caspase-8 from Alexis Corp.

Cell lines, primary samples, and amplification in NOD/SCID mice, cell death assays

All parental cell lines were obtained from DSMZ, CEM-TR and J-TR cells had been described previously (11, 16). After thawing, parental cells were amplified and numerous aliquots were frozen within 1 month after thawing of original cells. Each frozen aliquot was then rethawn and used for a maximum of 3 months after thawing. Derivative cells stably transfected with short hairpin (shRNA) against caspase-8 or p53 have been published before and cells were maintained as described there (11, 16).

For all cell line experiments, cells were seeded as described before and incubated with cytotoxic drugs for 48 hours (11). For combined stimulation, cells were incubated with MTX for 48 hours, followed by the second stimulant for additional 48 hours. Cell death was measured by forward side scatter analysis using FACscan (Becton Dickinson). For biochemical inhibition of caspase-8 or death receptor blockade, cell lines were pretreated for 6 hours before stimulation.

Primary leukemia blasts were investigated from $n = 29$ children treated for acute leukemia at the Ludwig Maximilians University's children's hospital and the children's hospital of the TU Munich during 2005 and 2008. All experiments were approved by the local ethics boards according to the declaration of Helsinki. Samples were obtained, isolated, and seeded as described (11). Cells were stimulated simultaneously with MTX (30 μmol/L) and TRAIL (1 μg/mL) or cytotoxic drugs at peak plasma concentration as described previously (22). TRAIL resistance was defined as specific cell death by TRAIL less than 10%. For molecular studies, primary cells were xenografted in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice and amplified before molecular modulation.

Western blot analysis

Western blot analysis of total cellular protein was done for all cell line experiments as described (16). For investigation of primary samples, Iceman's lysis buffer (Tris HCl 10 mmol/L pH 7.5, NaCl 50 mmol/L, Triton X-100 0.5%, desoxycholic acid 0.5%, and sodium duodecyl sulfate 0.5%) was used. For quantification of caspase-8 expression in primary samples, AIDA Image Analyzer was applied.

Transfection experiments

In addition to formerly described stably transfected cell lines, parental CEM and JURKAT cells were stably transfected with previously specified vectors expressing shRNA against caspase-8 or a mock sequence using the Amaxa Cell Line Nucleofactor Kit V (Lonza) according to the manufacturers' instructions (11). As alternative shRNA target for caspase-8, the following sequence was studied: 5′-TCTATTAATTCGGAAGAGC-3′. Primary samples were transfected using the identical transfection technique as for the cell lines and the following siRNAs at a concentration of 20 μmol/L: silencer validated siRNA-p53 (5′-GGGUUAGUUUACAAUCAGC-3′; Ambion), siRNA-Caspase-8 (5′-GCUCUUCCGAAUUAAUAGATT-3′) and as control sequence siRNA-Lamin (5′-ACUGCAGCAUCAUGUAAUCTT-3′, both from Eurofins MWG Operon). Primary cells were resuspended in RPMI medium supplemented with 20% fetal calf serum, 1% penicillin/streptomycin, 1% gentamycin, 6 μL/mL mixture

of insulin, transferrin, and selenium (Invitrogen), 1 mmol/L sodium pyruvate and 50 μmol/L 1-thioglycerole. Twelve hours after transfection, cells were simultaneously stimulated with MTX and cytotoxic drugs for 48 hours. Efficacy of inhibition of caspase-8 upregulation by siRNA interference after MTX was shown by Western blot analysis at the end of total incubation time for each experimental setting.

Statistical analysis

Normalized isobolograms were generated using CompuSyn software. For cell line experiments, data are presented as mean values of at least 3 independent experiments ± SEM if not stated differently. To test for statistically significant differences, 1-way RM ANOVA or paired *t* test was applied. Primary samples were evaluated using 1-way RM ANOVA on ranks or Spearman's rank order correlation test. Statistical significance was accepted with *P* values less than 0.05.

Results

Caspase-8 plays a pivotal role for apoptosis induction via the extrinsic apoptosis signaling cascade, although its role for intrinsic apoptosis signaling induced by cytotoxic drugs seems tumor type specific (20, 21). Here, we characterized the role of caspase-8 for cell death induction by cytotoxic drugs in B- and T-ALL cells.

Cytotoxic drug-induced cell death depends on caspase-8

The T-cell ALL cell lines CEM and JURKAT showed abundant expression of caspase-8 and certain sensitivity toward cell death induction by numerous cytotoxic drugs of current antileukemia treatment protocols, except dexamethasone. Upon induction of cell death, all cytotoxic drugs induced activation of p53 (Supplementary Fig. S1A) and cleavage of caspase-8 (Supplementary Fig. S1B).

To study the causative role of caspase-8 for mediating cell death induction by cytotoxic drugs, we compared parental T-ALL CEM and JURKAT cells with several derivative cell lines. In the derivative cell lines, expression of caspase-8 was downregulated either by stable expression of a small hairpin RNA directed against casaspe-8 or by epigenetic silencing of the caspsae-8 promoter (11, 16, 23). Caspase-8 low-expressing ALL cell lines are known to be resistant against cell death induction by TRAIL and CD95L (11) and partially resistant toward daunorubicin and camptothecin-induced cell death (21). In addition to these known agents, downregulation of caspase-8 disabled effective cell death induction by the majority of routinely used cytotoxic drugs including asparaginase, cyclophosphamide, doxorubicin, 6-thioguanine, and vincristine (Fig. 1A, Supplementary Fig. S1C and D). In this experiment, CEM-TR cells were more resistant toward drug-induced apoptosis compared with CEM cells stably expressing the shRNA against caspase-8 eventually because of the incomplete knockdown in the latter as observed in Western blot. In addition to T-ALL cell lines, the B-ALL lines BJAB, SEM, and REH were tested and

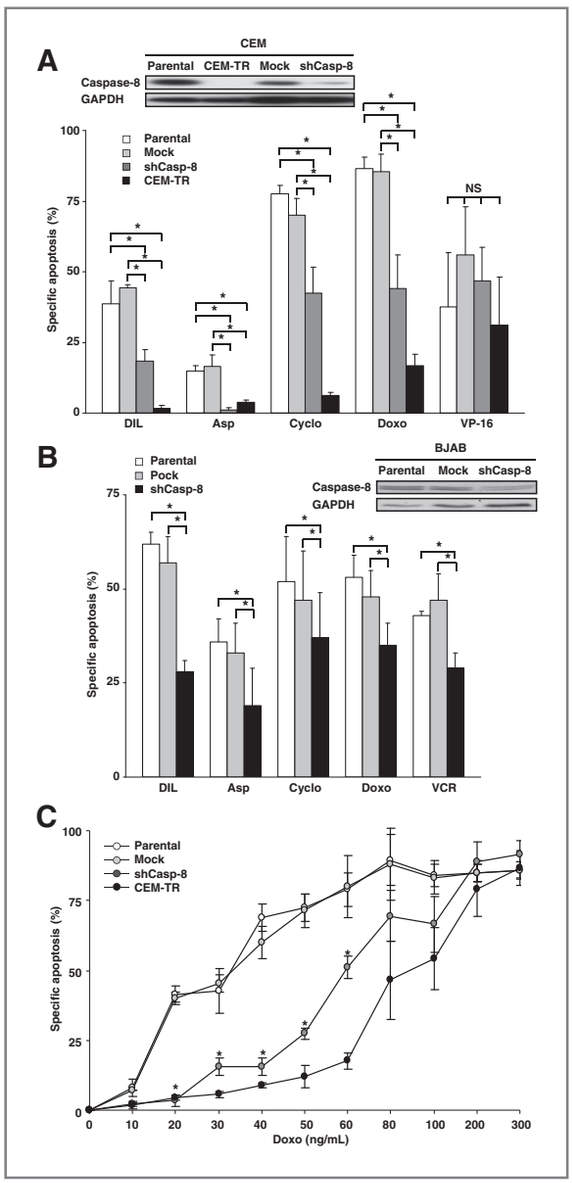

Figure 1. Enhanced cell death induction by cytotoxic drugs in the presence of caspase-8 in leukemia cell lines. A, parental CEM cells were stably transfected with shRNA against Casp-8 (shCasp-8) or a mock sequence (mock) and Western blot analysis was done of total cellular protein including derivate CEM-TR cells with permanent downregulation of caspase-8 because of epigenetic regulatory mechanisms. Cells were stimulated for 48 hours with death inducing ligand TRAIL (300 ng/mL) together with Apo-1 (1 μg/mL) and protein A (5 ng/mL; DIL), asparaginase (Asp, 1 U/mL), 4-hydroperoxy-cyclophosphamide (Cyclo, 0.3 μmol/L), doxorubicin (Doxo, 100 ng/mL), or etoposide (VP-16, 0.3 μmol/L) for 48 hours. *, comparing parental or mock-transfected cells with cells transfected with shCasp-8 or TR cells. B, BJAB cells stably transfected with shRNA against Casp-8 (shCasp-8) or a mock sequence (mock) were stimulated with DIL, Asp, Cyclo, Doxo, and vincristine (VCR) and analyzed as in Fig. 1A and Supplementary Fig. S1D. Doxo and VCR were applied at 30 ng/mL. C, CEM cells from Fig. 1A were stimulated with Doxo for 48 hours as indicated. Cell death was measured using forward side scatter analysis. Data are presented as mean ± SEM of at least 3 independent experiments. *, $P < 0.05$, ANOVA, comparing parental and mock cells to shCasp8 and CEM-TR cells. NS, not significant.

all showed certain dependency on caspase-8 for drug-induced apoptosis, although to a minor extent than JURKAT and CEM cells (Fig. 1B, Supplementary Fig. S1E and data not shown). Unfortunately, corticoids could not be studied in this setting, because of complete apoptosis resistance (data not shown). Thus, the majority of cytotoxic drugs used in the treatment of ALL depended on the presence of caspase-8 for effective cell death induction.

In line with these results, biochemical inhibition of caspase-8 activation by the caspase-8 directed inhibitor zIETD or a second independent shRNA against caspase-8 inhibited cell death induction by the same drugs (Supplementary Fig. S1F and G and data not shown). Upon downregulation of caspase-8, high concentrations of drugs, such as doxorubicin, were required for efficient cell death induction, although these concentrations are measured only in some patients and for short periods of time, although clinically more relevant concentrations were less effective in cells with downregulated caspase-8 (Fig. 1C and data not shown; refs. 22, 24).

Thus, caspase-8 represents an important intracellular signaling mediator for a high number of cytotoxic drugs used in routine antileukemia treatment.

Caspase-8 is downregulated in a subset of primary ALL cells

As we found an important role of caspase-8 for drug-induced cell death in ALL cell lines, we next investigated the caspase-8 expression levels in primary ALL cells. In many solid tumor entities such as neuroectodermal tumors, downregulation of caspase-8 is a frequent event in patients' tumors associated with drug resistance and poor prognosis (2, 5, 8). In line, high expression levels of caspase-8 mRNA were prognostically significant for favorable response to initial chemotherapy in childhood ALL (7, 8).

To further clarify the role of caspase-8 for efficient apoptosis induction, we determined the expression level in 29 primary childhood ALL samples derived from bone marrow aspiration before onset of therapy using Western blot analysis standardized to GAPDH (Supplementary Fig. S2). Twenty-four of 29 (83%) samples showed expression levels of caspase-8 lower than that in JURKAT cells with apparently undetectable expression of caspase-8 in 6 of 29 (21%) of samples. Clinical parameters were available for 4 of the 6 patients with ALL cells showing apparently absent expression of caspase-8. Interestingly, all 4 patients had enhanced clinical risk factors, 3 of 4 showing high risk criteria at primary disease and 1 patient with ALL relapse. These 6 samples showed complete resistance to extrinsic cell death signaling, for example, by the death inducing ligand TRAIL (data not shown).

In addition to the determination of caspase-8 protein levels, primary ALL cells were screened for *in vitro* cell death sensitivity against a subset of cytotoxic drugs. Despite of low sample numbers, expression levels of caspase-8 significantly correlated to *in vitro* cell death induction by doxorubicin and dexamethasone (Fig. 2A and B) and to some extent to 6-thioguanin ($P = 0.087$). In contrast, cell death induction by cytarabine and MTX, which showed caspase-8–independent cell death in cell line experiments (Fig. 1), were completely independent from caspase-8 levels ($P = 0.96$ for cytarabine and $P = 0.58$ for MTX).

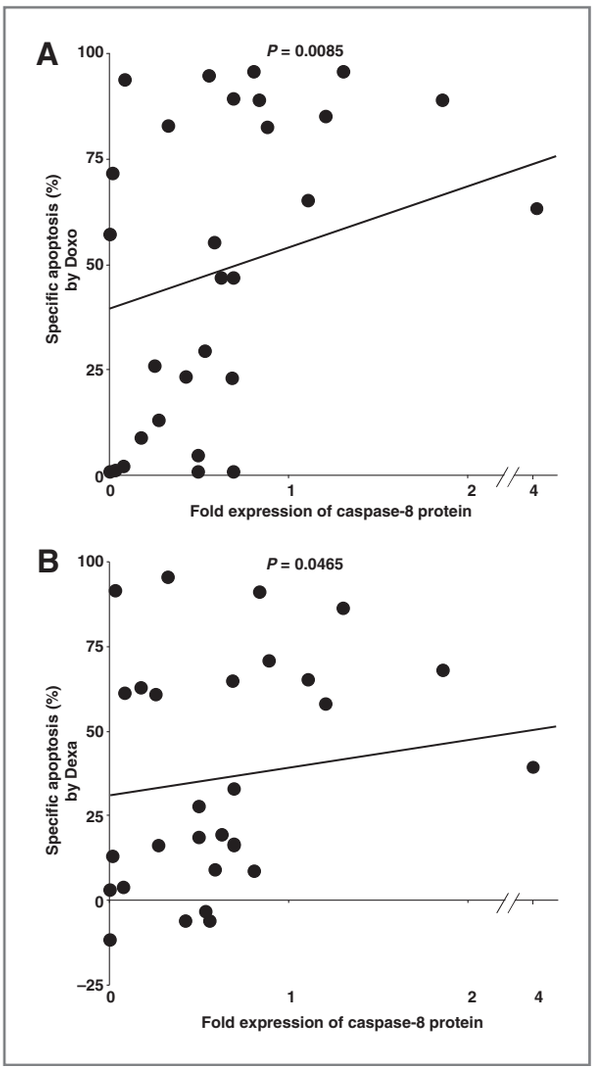

Figure 2. Correlation between caspase-8 expression and drug sensitivity in primary leukemia cells. A and B, $n = 29$ primary childhood acute leukemia samples were evaluated for caspase-8 expression on protein level by Western blot analysis. Caspase-8 expression was standardized to GAPDH and is depicted as relative expression as shown in detail in Supplementary Fig. S2. Relative caspase-8 expression was correlated to cell death induction by Doxo (A; 0.5 μmol/L; $P = 0.0085$; $r = 0.481$) or dexamethasone (Dexa; B; 10–5 mol/L; $P = 0.0465$; $r = 0.377$). Statistical analysis was done using Spearman's rank order correlation test. r = correlation coefficient.

The data showed that downregulation of caspase-8 protein is not restricted to solid tumor cells but also occurs in a relevant fraction of ALL samples. Downregulation of caspase-8 leads to apoptosis resistance of primary ALL cells toward defined routinely used cytotoxic drugs *in vitro*.

Cytotoxic drugs induce cell death independently from death receptor signaling

During cell death signaling, caspase-8 can be activated by 2 different mechanisms: Upon extrinsic apoptosis signaling, caspase-8 becomes activated in the proximal cell death signaling pathway at the death receptors. Within the intrinsic apoptosis signaling pathway, caspase-8 is activated downstream of mitochondria by executioner caspases to fulfill an amplifier function (11, 19). During drug-induced cell death, the activation of the death receptor system has been controversially discussed (25–27).

To discriminate, whether caspase-8 becomes activated by the extrinsic or the intrinsic apoptosis signaling pathway upon stimulation by cytotoxic drugs in ALL cells, death receptors were blocked by inhibitory antibodies. These antibodies were able to completely block cell death induction by death-inducing ligands (Fig. 3). Nevertheless, death receptor blockade did not alter cell death induction by cytotoxic drugs (Fig. 3 and Supplementary Fig. S3A and B), arguing toward a death receptor–independent activation of caspase-8 in our model of CEM and JURKAT T-cell leukemia cells.

MTX upregulates epigenetically downregulated caspase-8

So far, we have shown that caspase-8 plays an important role for cell death induction by a number of cytotoxic drugs in ALL cells (Fig. 1 and 2). Surprisingly, a relevant subset of primary ALL cells expressed only low amounts of caspase-8 protein followed by reduced *in vitro* sensitivity for cytotoxic drugs (Fig. 2). As patients carrying these tumor cells might be of high risk for poor response to initial polychemotherapy (7, 8), they might benefit from strategies restoring the expression of caspase-8 protein. Therefore, we next asked, whether apoptosis resistance against cytotoxic drugs that depend on functional caspase-8 could be overcome by upregulation of epigenetically downregulated caspase-8.

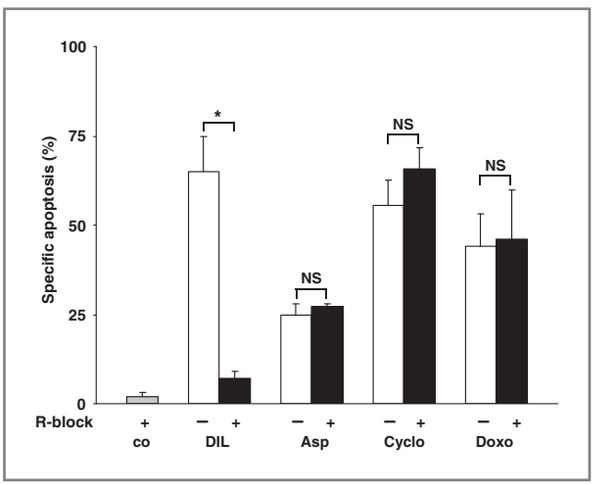

Figure 3. Activation of caspase-8 during drug-induced apoptosis independent of death receptors. Parental CEM cells were pretreated with Fas-blocking antibody (1 μg/mL) and DR4 and DR5 Fc fragments (1 μg/mL each; R-block) for 6 hours followed by stimulation with cytotoxic drugs as in Fig. 1A. Apoptosis measurement, presentation, and statistical analysis were done as in Fig. 1. *, $P < 0.05$, paired t test, comparing stimulated cells pretreated or not with the R-block.

As a first model, we used our derivative CEM and JURKAT cell lines with low expression of caspase-8, which were generated by weekly incubation of JURKAT and CEM cells with increasing sublethal doses of TRAIL over 30 weeks. These cells upregulated expression of caspase-8 by stimulation with the demethylating agent azacythidine (11, 16). We had shown previously that MTX upregulated the expression of caspase-8 protein by a mechanism dependent on the transcription factor p53 and thereby sensitized these resistant ALL cells toward TRAIL-induced apoptosis (ref. 11; Fig. 4C and D, Supplementary Fig. S4C and D, and data not shown). Here, we investigated whether further cytotoxic drugs were able to upregulate the expression of caspase-8. None of the drugs tested upregulated expression of caspase-8 except MTX (Supplementary Fig. S4A).

Upregulation of caspase-8 enables cell death induction by cytotoxic drugs

When the treatment of MTX was combined with doxorubicin, asparaginase, or cyclophosphamide, highly synergistic apoptosis induction was detected using normalized isobolograms (Fig. 4A and B, Supplementary Fig. S4B, and data not shown). Interestingly, MTX-mediated upregulation of caspase-8 enabled significant cell death induction by the caspase-8–dependent drugs tested (Fig. 4C and D and Supplementary Fig. S4C and D). To prove the role of caspase-8 for this cell death, MTX-induced upregulation of caspase-8 was inhibited by shRNA directed against either caspase-8 or p53. If upregulation of caspase-8 was inhibited on a molecular level, MTX was no longer able to sensitize ALL cells toward apoptosis induction by the caspase-8–dependent drugs (Fig. 4C and D and Supplementary Fig. S4C and D).

These data showed that upregulation of epigenetically silenced caspase-8, for example, by MTX overcomes apoptosis resistance of ALL cells against cytotoxic drugs with caspase-8–dependent signaling, such as asparaginase, cyclophosphamide, and doxorubicin.

In patient-derived ALL cells, upregulation of caspase-8 sensitizes for drug-induced apoptosis

ALL cell lines frequently show mutations in p53, although primary ALL cells rarely do so. To avoid working on cell line artefacts and to approximate the clinical situation, we investigated upregulation of caspase-8 in our primary ALL samples with low expression of caspase-8. We used MTX for upregulation of caspase-8 and the 6 samples from Fig. 2 with lowest expression of caspase-8. MTX induced marked upregulation of caspase-8 in all 6 primary ALL samples (Fig. 5A).

We next aimed to prove a causative role of p53-mediated regulation of caspase-8 to enable apoptosis induction by caspase-8–dependent drugs by MTX in primary ALL cells. Unfortunately, the small sample volumes of these cells disabled performing molecular modulations in primary samples. To remain as close as possible to the characteristics

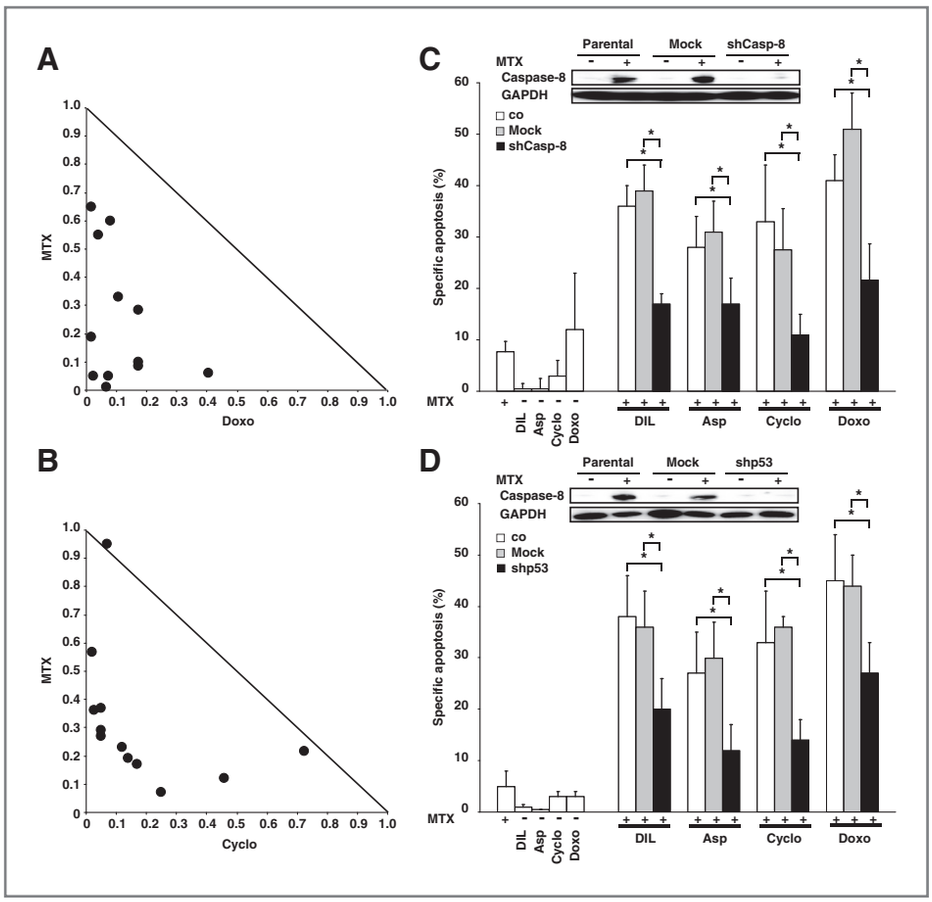

Figure 4. MTX-mediated sensitization for drug-induced cell death by p53-dependent regulation of caspase-8 in leukemia cells. A and B, CEM-TR cells were stimulated with MTX (10, 30, 100, and 300 nmol/L) for 48 hours followed by Doxo (10, 30, 100, and 300 ng/mL; A) or cyclo (0.1, 0.3, 0.6, and 1 μmol/L; B) for another 48 hours. The effect of the combined stimulation is presented using normalized isobolograms. C and D, CEM-TR cells stably transfected with shRNA against caspase-8 (shCasp-8; C) or with shRNA against p53 (shp53; D), a mock sequence (mock) or left untreated (co) were pretreated with MTX (6 nmol/L) for 48 hours followed by incubation with death-inducing ligand TRAIL (300 ng/mL) together with Apo-1 (1 μg/mL) and protein A (5 ng/mL; DIL), Asp (1 U/mL), cyclo (0.3 μmol/L), or Doxo (100 ng/mL) for an additional 48 hours. In parallel, Western blot analysis was done of unstimulated and MTX-treated cells. For ease of reading, apoptosis induction by single agents is presented only for parental cells but did not differ from cell death induced in derivative cells. Cell death measurement, statistical analysis, presentation of data, and Western blot were done as in Fig. 1. *, $P < 0.05$, ANOVA, comparing the effect of the combinatorial treatment on parental cells and cells transfected with the mock sequence or the specific shRNA.

of primary ALL cells, we amplified them in mice using the xenograft NOD/SCID mouse model (28). For molecular experiments, we used our recently established technique allowing electroporation-based transient transfection of siRNA into patient-derived ALL cells freshly isolated from mice. In contrast to CEM and JURKAT cells, these cells were not completely resistant against corticoid-induced apoptosis, enabling additional studies on dexamethasone-induced apoptosis.

To study the impact of p53-mediated upregulation of caspase-8 on a molecular level, mouse-amplified ALL cells from 2 different patients with low constitutive expression of caspase-8 (#6, pre-B-ALL, initial diagnosis, female, 7 years and #4, pre-B-ALL, initial diagnosis, female, 5 years) from Fig. 5A were transfected with siRNA against caspase-8 or p53; this transfection prevented upregulation of caspase-8 upon stimulation with MTX (Fig. 5B and C). On a functional level, transient transfection with siRNA against caspase-8 or p53 diminished or completely prevented that MTX increased apoptosis by drugs dependent on caspase-8 such as TRAIL, asparaginase, cyclophosphamide, dexamethasone, or doxorubicin (Fig. 5B and C). Interestingly, dexamethasone-induced cell death also depended on expression levels of caspase-8, adding dexamethasone to the list of caspase-8–dependent routine drugs of ALL.

Taken together, caspase-8 represents an important mediator for apoptosis induction by most, but not all drugs of current treatment of ALL. MTX upregulates expression of caspase-8 via p53 and thereby sensitizes for cell death induction by caspase-8–dependent drugs (Fig. 5D). Regulation of caspase-8 might represent a possible explanation as to why caspase-8–dependent drugs are particularly effective when combined with MTX, as realized in clinical treatment protocols of ALL since decades.

Discussion

The molecular studies unravel a surprisingly important function of caspase-8 during apoptosis induction by cytotoxic drugs in these cells. The relevance of caspase-8 for effective cell death induction was clarified for 2 different situations: (i) In B- and T-ALL cell lines and primary B- and T-ALL cells with basic expression of caspase-8, efficiency of cell death induction by the majority of routine cytotoxic drugs depended on caspase-8. (ii) In the subset of ALL cells with downregulated caspase-8 because of epigenetic regulatory mechanisms, MTX was able to upregulate caspase-8. Here, the efficiency of defined drug combinations involving MTX was coupled to the reexpression of caspase-8.

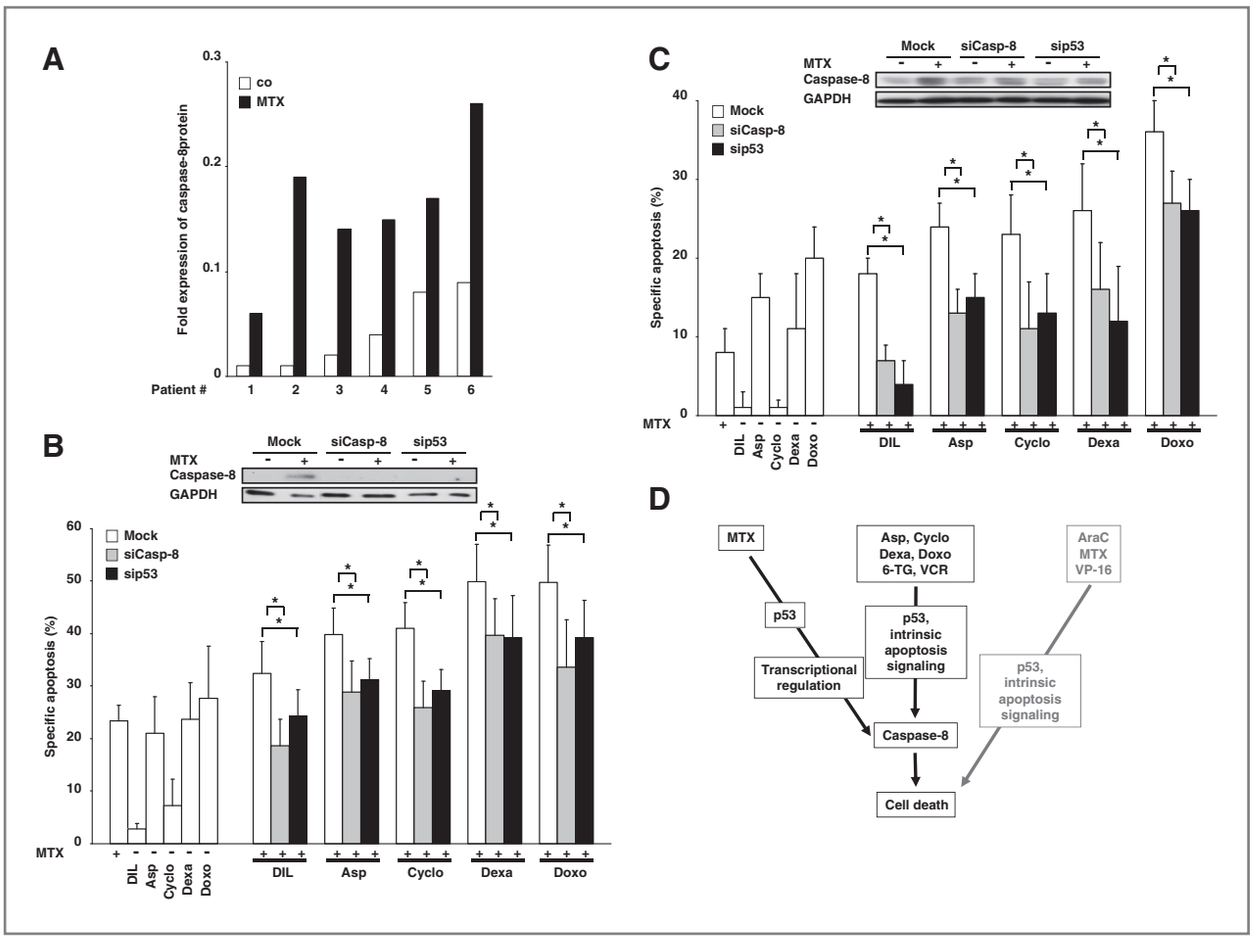

Figure 5. Augmented cell death induction by cytotoxic drugs in the presence of caspase-8 in patient-derived acute leukemia cells. A, the 6 primary samples with downregulated caspase-8 from Fig. 2 were left untreated or stimulated with MTX (30 μmol/L) for 48 hours and analyzed as in Fig. 2. B, primary cells from sample #6 (pre-B-ALL, initial diagnosis, female, 7 years) from Fig. 5A with downregulated caspase-8 were amplified in NOD/SCID mice and transfected with a nonspecific sequence (mock) or siRNA against caspase-8 (Casp-8) or p53. After 12 hours, cells were simultaneously stimulated with MTX (30 μmol/L) and TRAIL (1 μg/mL), Asp (0.3 U/mL), Cyclo (0.3 μmol/L), Dexa (10^{-9} mol/L), or Doxo (0.05 μmol/L) for 48 hours. In parallel, Western blot analysis was done of cells incubated with MTX for 48 hours. Depicted are mean values from $n = 6$ independent experiments. Statistical analysis was done comparing cell death induction in samples transfected with a mock sequence or siRNA against Casp-8 or p53 for each stimulatory setting, $P < 0.05$, 1-way RM ANOVA on ranks. NS, not significant. C, primary cells from sample #4 (pre-B-ALL initial diagnosis, female, 5 years) from Fig. 5A with downregulated caspase-8 were obtained, transfected, stimulated, and analyzed as in Fig. 5B. Depicted are mean values of $n = 9$ independent experiments. D, cytotoxic drugs investigated were classified according to their ability to activate p53, to regulate the expression of caspase-8, and their dependency of efficient cell death induction on the presence of caspase-8. Cell death measurement and Western blot were done as in Fig. 1 and presentation of data as in Fig. 4 if not stated differently. *, $P < 0.05$, ANOVA, comparing the effect of the combinatorial treatment on cells transfected with the mock sequence or the two different specific siRNAs.

The role of caspase-8 for chemosensitivity was shown in (i) primary tumor cells from children with ALL; (ii) primary ALL cells after amplification in NOD/SCID mice, and (iii) ALL cell lines. Using modern molecular technologies, we confirmed a causative role of caspase-8 on patient-derived ALL cells and provided the molecular proof for the clinically observed association between polychemotherapy efficiency and caspase-8 expression (8).

Whereas a role of caspase-8 was known only for daunorubicin and camptothecin-induced cell death so far (21), our data broadly widen the number of clinically used cytotoxic drugs which depend on caspase-8 for induction of apoptosis. The downstream amplifier function of caspase-8 in the intrinsic apoptosis signaling pathway is thus highly important for chemotherapy in ALL cells, in contrast to, for example, Ewing sarcoma cells (20). Our data represent the first molecular studies on patient-derived ALL cells on the role of caspase-8 for apoptosis induction by routine cytotoxic drugs.

From the 9 drugs studied, 6 drugs depended on caspase-8 for induction of apoptosis. For yet unknown mechanisms, cytarabine, etoposide, and MTX activated caspase-8, but induced apoptosis independently from caspase-8 (Fig. 5D). Routinely, cytotoxic drugs are classified regarding their

main mechanism of action. Whereas, for example, the topoisomerase inhibitor doxorubicin induced caspase-8–dependent cell death, the topoisomerase inhibitor etoposide did not. No correlation was detected between the mechanism of action of the drugs used and their dependence on caspase-8 for induction of apoptosis. Additional studies are required to clarify how dependency of caspase-8 is related to the classifying mechanism of action of cytotoxic drugs.

Since decades, MTX is successfully used in combination with nearly all drugs used in polychemotherapy protocols of ALL (29–31). Our data show that a possible explanation for the favorable effects of some of these drug combinations might rely on regulation of caspase-8. In addition, MTX sensitized for apoptosis induction by drugs signaling caspase-8–independent cell death. MTX sensitized ALL cells for cytarabine and etoposide by a caspase-8 and p53-independent mechanism (data not shown). To our surprise, we were not able to identify a specific group of p53 regulators able to restore caspase-8 within our experimental setting, but MTX was the only drug besides the formerly described 5-FU (11, 32).

More recently, it has become increasingly clear that antitumor therapy has to target epigenetic tumor alterations (33, 34). Caspase-8 is one of the frequently downregulated proteins due to epigenetic silencing (1, 3, 10). We can show here that MTX, which is part of antileukemia protocols for decades, is able to upregulate epigenetically downregulated caspase-8 and thereby sensitizes tumor cells toward drug-induced apoptosis. Thus and without awareness of the underlying mechanisms, current polychemotherapy protocols obviously target epigenetic tumor alterations. The old well known MTX is identified here as acting like a modern, epigenetic drug.

On a more general level, our work encourages the future search for targeted drug combinations which are based on the molecular understanding of the underlying signaling mechanism. Cytotoxic drugs that regulate caspase-8 should be combined with drugs that depend on the expression of caspase-8. These drug combinations inherit the potential of higher antitumor efficiency compared with empirically determined drug combinations. This approach is in line with current concepts of targeted therapies and the reevaluation of drug combinations (35–37). The current search of targeted therapeutics should be accompanied by a search for targeted drug combinations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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