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Chemotherapeutic drugs sensitize pre-B ALL cells for CD95- and cytotoxic T-lymphocyte-mediated apoptosis

C Posovszky[1], C Friesen[2], I Herr[1] and K-M Debatin[1,2]

[1]*Division of Molecular Oncology, German Cancer Research Center, Heidelberg; and* [2]*University Children's Hospital, Ulm, Germany*

The CD95 (APO-1/Fas) system plays an important role in lymphocyte homeostasis and contributes to anticancer drug-induced apoptosis in some tumor cells. Most childhood B-lineage ALL cells are constitutively resistant towards CD95-induced death. We report here that chemotherapeutic drugs, such as doxorubicin, cytarabine, methotrexate and 6-mercaptopurine, sensitize CD95-resistant pre-B-ALL cell lines for CD95- and lymphokine-activated killer (LAK)-induced cell death. Enhanced susceptibility in drug-treated cells was found to be associated with increased expression of CD95 mRNA and surface expression of CD95 protein, as well as loss of Bcl-x_L protein and disturbance of mitochondrial function. Low level activation of caspases and cleavage of poly(ADP-ribose) polymerase following CD95 triggering was strongly increased in drug pre-treated cells. Furthermore, drug pre-treated cells could be rescued from CD95-mediated apoptosis by blocking the CD95-signaling pathway with a FADD-dominant negative expression construct. Our data suggest that chemotherapeutic drugs may sensitize pre-B ALL cells by at least two mechanisms: (1) by increasing CD95 expression; and (2) by lowering the threshold for apoptotic signals. These findings may explain the effectiveness of low-dose chemotherapy and indicate an active role for key molecules of apoptosis and the immune system during chemotherapy of leukemia.
Keywords: CD95 (APO-1/Fas); apoptosis; cytotoxic drugs; B-lineage ALL

Introduction

Chemotherapeutic agents used in the treatment of childhood acute lymphoblastic leukemia (ALL) have been identified to kill susceptible cells by apoptosis.[1–3] After DNA damage or metabolic inhibition in target cells, cytotoxic drugs induce a common apoptosis signaling pathway which leads to proteolytic cleavage of poly(ADP-ribose) polymerase and subsequent DNA fragmentation.[4] However, the intracellular mechanisms by which the specific damage induced by chemotherapeutic agents are converted into death signals are not entirely understood.

One possible mechanism which may mediate drug injury in T cell leukemias is the increase in expression of the physiological death inducing CD95-ligand (CD95-L) after treatment with doxorubicin or methotrexate.[5] Interaction of CD95-L with its receptor CD95 plays a major role in lymphocyte homeostasis and growth control.[6] The 48 kDa CD95 surface antigen can mediate apoptosis upon crosslinking with monoclonal antibodies (anti-Fas, anti-APO-1) or natural cognate CD95-L.[7–9] Many cell types, both normal and malignant cells, express CD95.[10–15] While some T- and B-lineage malignancies are sensitive to CD95 triggering, the majority of primary leukemias are resistant towards CD95-mediated apoptosis.[8,15,16]

Triggering of the T cell receptor in peripheral T cells leads to activation-induced cell death via CD95/CD95-L interaction.[17] Similarly, antigen-activation in B cells leads to upregulation of CD95 and sensitivity towards CD95-induced apoptosis.[18] Apart from immune activation, cytotoxic drugs are also able to stimulate the CD95 system. Upregulation of CD95 and CD95-L was observed in T-ALL, neuroblastoma and hepatoma cells.[5,19,20] This mechanism may account for the sensitivity of these cells towards chemotherapy. In drug-resistant and CD95-resistant cell lines with a crossresistant phenotype, a downregulation of CD95 compared to parental cell lines was found.[21,22] Lymphokine-activated killer (LAK) cells use CD95-L, perforin and TNFα to mediate cytotoxicity by induction of apoptosis in target cells.[23,24] Consequently, drug- and CD95-resistant cell lines were found to be less susceptible to LAK cell kill than the sensitive parental cell lines.[25]

Interestingly, combination treatment with anticancer drugs and agonistic anti-CD95 antibodies caused a superadditive apoptosis-promoting effect in leukemic, glioma or colon carcinoma cells[26–28] and drug-resistant tumor cell lines which were crossresistant against CD95 triggering became highly sensitive towards CD95-induced apoptosis.[29]

In the present study, we examined the drug-induced mechanisms of sensitization to LAK cell- and CD95-mediated apoptosis in pre-pre and pre-B ALL cell lines. We found that cytotoxic drugs increase apoptosis sensitivity by influencing CD95 expression, caspase activity, mitochondrial function and expression of Bcl-2 family members.

Materials and methods

Cell lines and culture conditions

CEM (T-ALL), REH (pre-pre B ALL, $CD10^+$, $CD19^+$, $CD20^+$, $clgM^-$), Nalm6 (pre-B ALL, $CD10^+$, $CD19^+$, $CD20^+$, $clgM^+$) and KM3 (B ALL, $CD10^-$, $CD19^+$, $CD20^+$, $slgM^+$) were grown in RPMI 1640 (Gibco BRL, Eggenstein, Germany) containing 10% FCS (Congo, Wiesbaden, Germany), 10 mm HEPES, pH 7.3 (Biochrom, Berlin, Germany), 200 U/ml penicillin-streptomycin (Gibco BRL) and 2 mm l-glutamine (Biochrom), further referred to RPMI*. Nalm6-RD, a doxorubicin-resistant cell line, was generated by continuous culture in medium containing 0.1 μg/ml doxorubicin for more than 3 months.

Drugs

Doxorubicin (Farmitalia, Milano, Italy), cytarabine (Mack, Illertissen, Germany), methotrexate (Lederle, Wolfratshausen, Germany) and 6-mercaptopurine (Deutsche Wellcome, Germany) were used. Doxorubicin and cytarabine were freshly dissolved in sterile distilled water and methotrexate, as well as 6-mercaptopurine, were freshly dissolved in 0.01 m NaOH.

Correspondence: K-M Debatin, Universitäts-Kinderklinik, Prittwitzstr 43, 89075 Ulm, Germany; Fax: 49 731 502 6681
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Preparation of LAK cells

Freshly drawn heparinized blood from healthy donors was diluted 1:3 with sterile phosphate buffer saline (PBS) and mononuclear cells (PBMC) were isolated by Ficoll–Paque density gradient centrifugation. LAK cells were prepared by culture of fresh PBMC at a concentration of 1×10^6 cells/ml RPMI* containing 500 U/ml interleukin (IL-)2 (Novus Molecular, San Diego, CA, USA). Cells were washed and resuspended in fresh medium containing IL-2 every 5 days. Cytotoxicity assays were performed starting at day 10. Before using them in the assay, LAK cells were separated from dead cells by Ficoll separation.

Determination of apoptosis

For quantitative determination of cell death we used the forward and side scatter profile and the annexin binding of apoptotic cells. Annexin V-solution was prepared by adding 2 μl annexin FITC (Bender Med Systems, Wien, Austria) to 100 μl of staining buffer containing Sterofundin (B Braun, Melsungen, Germany) supplemented with 0.5% bovine serum albumin (Sigma, Deisenhofen, Germany) and 10 mm HEPES (Biochrom, Berlin, Germany). Washed and pelleted cells were resuspended in 15 μl annexin V-solution and incubated in the dark for 45 min at 4°C. For LAK assays, cells were co-incubated with PE-conjugated CD19 antibodies (Dako, Hamburg, Germany). After incubation cells were washed and resuspended in 100 μl staining buffer for flow cytometry analysis. Annexin V binds to exposed phosphatidylserine on the surface of apoptotic cells.[30,31] Annexin V-FITC fluorescence as well as decreased forward and increased side scatter (FSC/SSC) profile of cells were measured with a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) using the Cell Quest software program (Becton Dickinson).

The extent of specific apoptosis (%) was calculated as described elsewhere:[16]

$$\frac{(\text{CD95- or LAK-mediated apoptosis} - \text{spontaneous apoptosis}) \times 100}{(100 - \text{spontaneous apoptosis})}$$

Detection of CD95 expression

For flow cytometric analysis, 5×10^5 cells were washed with PBS and incubated for 45 min at 4°C with either anti-APO-1 IgG1 supernatant[8] or IgG1 control antibody (Dako) both diluted in an antibody-diluting agent with background-reducing quality (Dako). After washing cells were incubated with goat-F(ab')$_2$ anti-mouse IgG (H + L) FITC labeled antibody (Immunotech, Hamburg, Germany) for 45 min at 4°C. Finally, washed cells were analyzed by flow cytometry.

RT-PCR for CD95, FADD and FLICE mRNA

Total RNA was prepared using the Qiagen total RNA kit (Qiagen, Hilden, Germany). RNA was converted to cDNA by reverse transcription and amplified for 35 cycles by PCR in a thermocycler (Stratagene, Heidelberg, Germany) using the Gene Amplification RNA-PCR core kit (Perkin-Elmer, Branchburg, NJ, USA) following the manufacturers' instructions. Primers used for amplification of CD95, FADD and FLICE mRNA were prepared (MWG-Biotech, Ebersberg, Germany) according to the following sequences: CD95 (forward 5′-TCA AGG AAT GCA CAC TCA CCA GC-3′ and reverse primer 5′-GGC TTC ATT GAC ACC ATT CTT TCG-3′); FADD (forward 5′-GGA GAA GGC TGG CTC GTC AGC TCA AA-3′ and reverse primer 5′-GGT CCA CCA GCG CAA AGC AGC-3′); FLICE (DED) (forward 5′-GGA CTA CAT TCC GCA AAG GAA GCA AG-3′ and reverse primer 5′-TGG GCA CAG ACT CTT TTC AGG ATG TC-3′); FLICE (CASP) (forward 5′-GGA CAG GAA TGG AAC ACA CTT GGA TG-3′ and reverse primer 5′-CTG GCA AAG TGA CTG GAT GTA CCA GG-3′). GAPDH was used as an internal standard for RNA integrity. PCR products were run on a 2% agarose gel stained with ethidium bromide and visualized by UV illumination.

Assessment of mitochondrial transmembrane potential $\Delta\psi_m$ and generation of reactive oxygen species (ROS)

The cationic lipophilic fluorochrome 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) (c = 460 ng/ml, Molecular Probes, Eugene, OR, USA) was used to measure $\Delta\psi_m$, whereas dihydroethidium (HE) (c = 126 ng/ml, Molecular Probes) was used to determine ROS generation. Cells were washed and incubated for 12 min at 37°C in the presence of the fluorochromes. After washing with PBS containing 1% FCS they were immediately analyzed by a FACScan flow cytometer (Becton Dickinson).

Western blot analysis

Cells were lysed in buffer containing 600 mm KCl, 20 mm HEPES-KOH, pH 7.9, 0.2 mm EDTA, 1 mm phenylmethylsulfonyl fluoride (Sigma) and 1 mm dithiothreitol (DTT) for 1 h on ice followed by high-speed centrifugation. The protein concentration in the supernatant was assayed using bicinchoninic acid (Pierce, Rockford, IL, USA). 50 μg of protein/lane were separated by 12% or 14% SDS-PAGE and electroblotted on nitrocellulose (Amersham, Braunschweig, Germany). Equal protein loading was controlled by Ponceau red staining of membranes. After blocking with 2% BSA (Sigma) and 0.1% Tween 20 (Sigma), immunodetection of FLICE, CPP32, PARP, Bcl-2 and Bcl-x was performed using anti-FLICE monoclonal antibody C15 (1:10 dilution of hybridoma supernatant), mouse anti-CPP32 monoclonal antibody (Transduction Laboratories, Lexington, KY, USA), rabbit anti-PARP polyclonal antibody (Boehringer Mannheim, Germany), rabbit anti-Bcl-x polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-Bcl-2 monoclonal antibody (Santa Cruz Biotechnology) and goat anti-mouse IgG or goat anti-rabbit IgG (Santa Cruz Biotechnology). An enhanced chemiluminescence system (Amersham) was used for detection.

Transient transfections

Cells in medium were pelleted and resuspended at 1×10^8 cells per 200 μl PBS with either 50 μg of FADD dominant-negative expression plasmid or empty pcDNA3 vector in presence of 5 μg GFP expression construct (Clontech). Transfection was done by electroporation (925 μF, 200 V). After transfection, cells were resuspended in fresh RPMI medium and

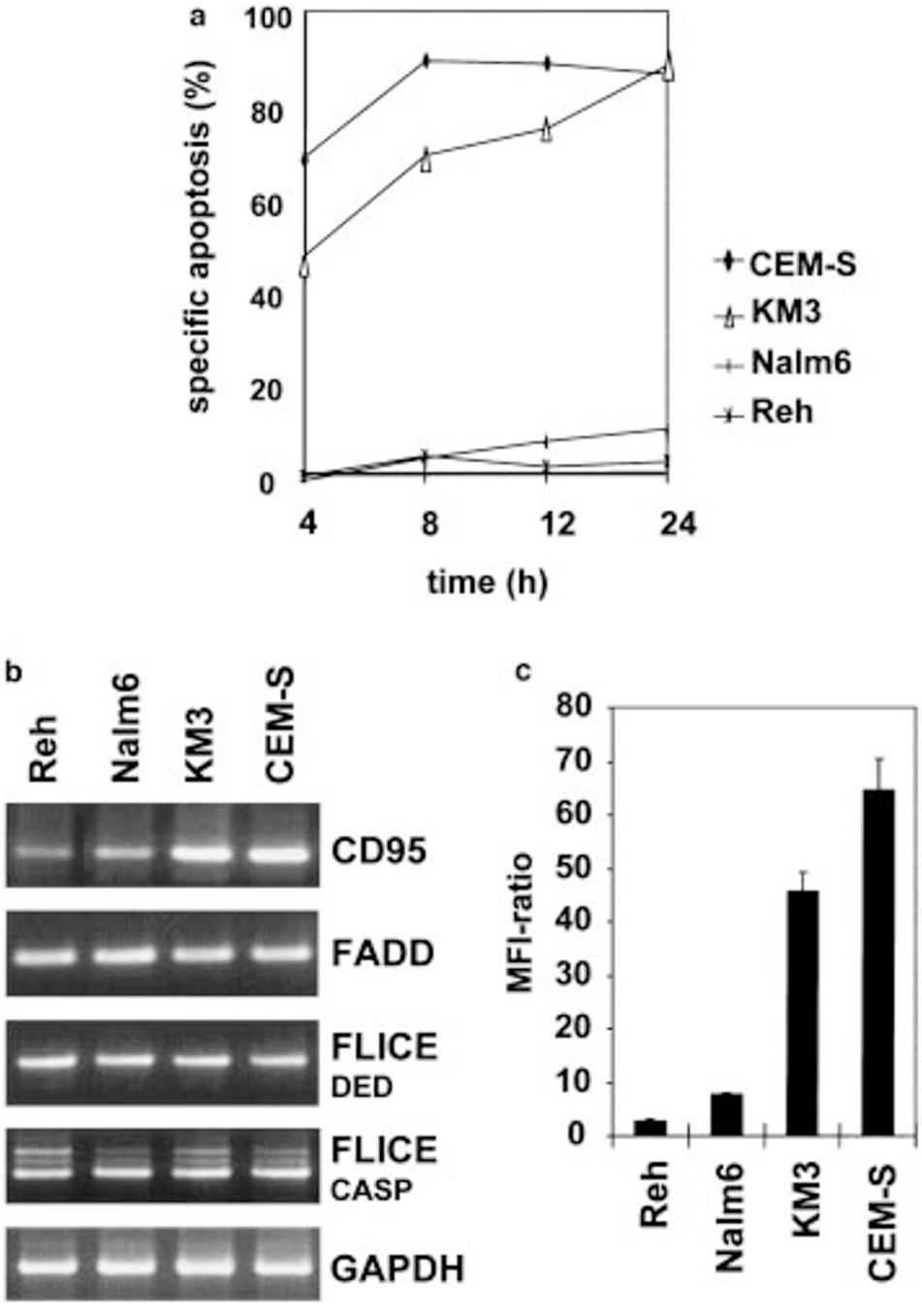

Figure 1 Susceptibility to anti-APO-1 induced cell death in ALL cell lines correlates with CD95 expression. (a) ALL cell lines (CEM-S, KM3, Nalm6 and REH) were treated with 1 μg/ml anti-APO-1 lgG3 antibody and 10 ng/ml protein A. After 4, 8, 12 and 24 h apoptosis was measured by analyzing FSC/SSC profile by flow cytometry. Specific apoptosis was calculated as described in Materials and methods. (b) Basal mRNA expression of CD95, FADD, FLICE (DED) N- and (CASP) C-terminus in CEM-S, KM3, Nalm6 and REH cells was examined by RT-PCR using sequence-specific primers. GAPDH served as a control for equal conditions. (c) CD95 surface expression of CEM-S, KM3, Nalm6 and REH cells was obtained by staining the cells with either anti-APO-1 supernatant or lgG1 isotype control antibodies and measuring FITC fluorescence of the secondary antibody by flow cytometry. MFl ratio was calculated as mean fluorescence of CD95-fluorescence divided through lgG1 isotype control fluorescence.

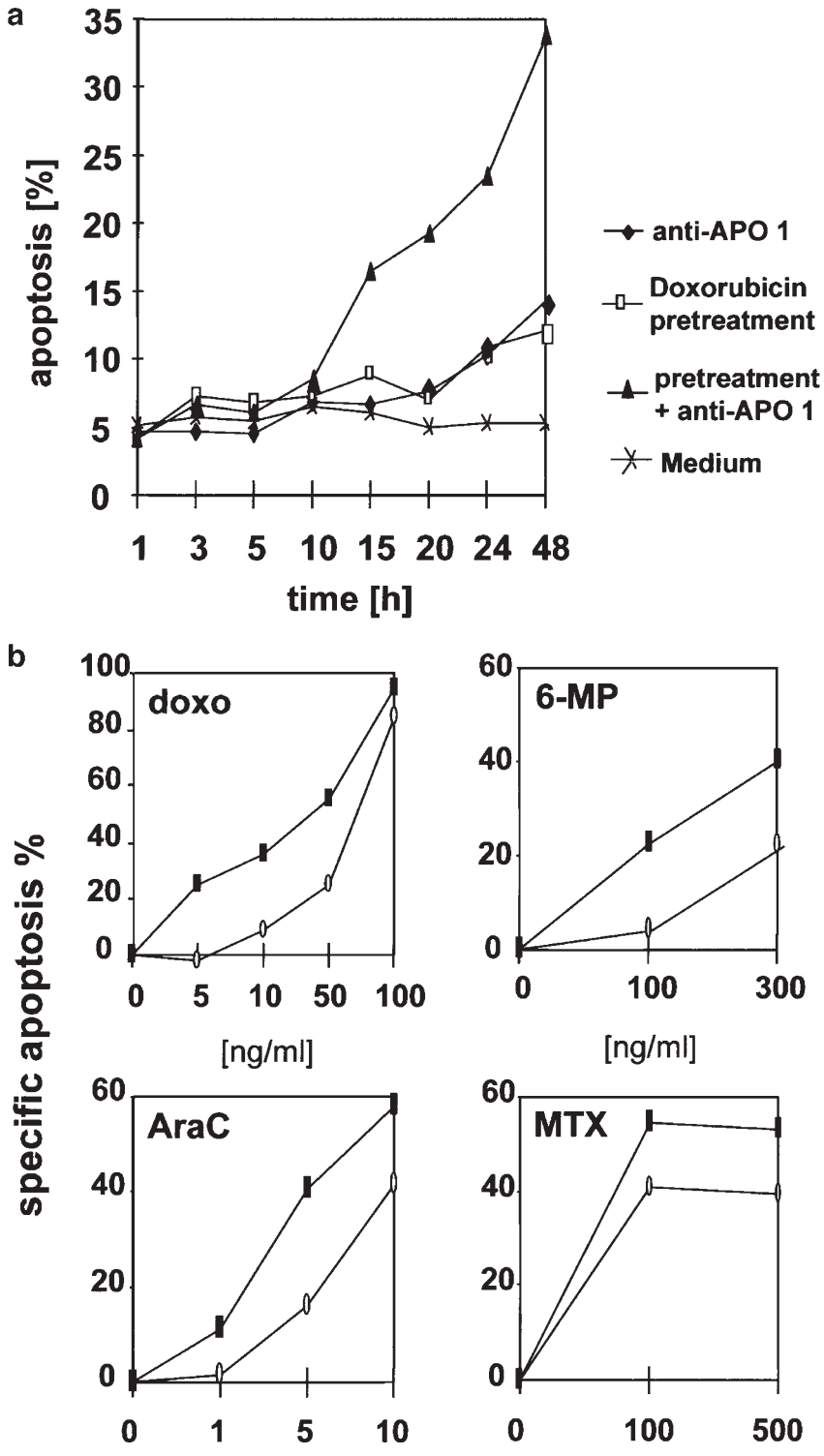

Figure 2 Cytotoxic drugs sensitize Nalm6 for CD95-mediated cell death. (a) Nalm6 cells were either left untreated or pre-treated with 0.01 μg/ml doxorubicin for 12 h. After washing the cells were divided and cultured in the presence of 1 μg/ml anti-APO-1 and 10 ng/ml protein A (filled symbols) or in medium alone (open symbols). 1, 3, 5, 10, 15, 20, 24 and 48 h later apoptosis was measured by analyzing FSC/SSC profile in flow cytometry. (b) The same procedure as in (a) was performed using different concentrations of doxorubicin, cytarabine, 6-mercaptopurine and methotrexate as indicated. Drug pre-treated cells were either incubated for 24 h with anti-APO-1 (filled square) or cultured in medium alone (open circle). FSC/SSC profile was analyzed by flow cytometry and specific apoptosis was calculated as described in Materials and methods.

cultured for 16 h. Prior to stimulation dead cells were separated by Ficoll and seeded in a 96-well flat-bottom plate.

Results

Precursor B-lineage ALL cell lines are resistant to anti-APO-1 mediated cell death

Despite the fact that most primary B-lineage ALL cells were found to be relatively resistant to CD95-induced cell death[15] no mutations in the CD95 molecule were found in 32 B-lineage ALL and five B-lineage ALL cell lines.[32] To further elucidate molecular mechanisms conferring resistance towards CD95 triggering we compared the highly anti-APO-1 sensitive cell lines CEM-S (T-ALL) and KM3 (B-ALL) and the resistant cell lines REH (pre-pre B-ALL) and Nalm6 (pre B-ALL) (Figure 1a) with regard to expression of molecules involved in the formation of the CD95 death inducing signaling complex (DISC)[33] including CD95, FADD and FLICE by RT-PCR (Figure 1b). Basal mRNA levels of CD95 were lower in resistant cell lines than in cell lines susceptible for anti-APO-1. This difference was confirmed by FACS analysis of the CD95 protein (Figure 1c). However, no difference between resistant and sensitive cells was found in mRNA expression levels of FADD and FLICE.

Drugs sensitize for CD95-mediated apoptosis

We recently described that CD95 receptor/ligand interactions are involved in doxorubicin- and methotrexate-induced apoptosis of human T-ALL cell lines.[5] Since Nalm6 and REH cells express low levels of CD95-L mRNA compared to CEM-S even after doxorubicin treatment or stimulation with phorbol myristate and ionomycine (data not shown, see also Ref. 34) we examined whether drug treatment would influence the susceptibility for CD95-triggered apoptosis. In order to mimic conditions of anticancer therapy *in vivo* we treated REH and Nalm6 cells with cytotoxic drugs for 12 h. Subsequently, cells were washed and incubated with anti-APO-1 antibody in drug-free RPMI for an additional 12 to 48 h at concentrations which correspond to plasma levels usually reached in standard treatment protocols for human ALL.[35–38] Surprisingly, the previously CD95-resistant cell lines Nalm6 and REH were rendered sensitive for anti-APO-1 induced cell death by pretreatment with doxorubicin (Figure 2a). Only cells treated with doxorubicin followed by anti-APO-1 displayed the typical morphological features of apoptosis, ie decreased forward scatter and increased side scatter (Figure 2a) as well as cell shrinkage, membrane blebbing and nuclear fragmentation (data not shown). In contrast, treatment with only one agent or culture in medium alone did not induce these changes (Figure 2a). Similar data were obtained in response to cytarabine, 6-mercaptopurine and methotrexate in Nalm6 (Figure 2b) and REH cells (data not shown).

Sensitivity to CD95-mediated apoptosis is blocked in FADD-DN cells

In order to ensure that the CD95 pathway is activated in cells pretreated with cytotoxic drugs we inhibited the death signal downstream of the CD95 receptor by transient transfection. Therefore we transfected the cells with a FADD-dominant negative (FADD-DN) expression construct or empty vector as control, together with a green fluorescent protein (GFP) construct to identify transfected cells. Anti-APO-1 alone did not induce apoptosis in any of the transfected cells. However, anti-APO-1-triggering of doxorubicin pre-treated cells failed to induce cell death in FADD-DN transfected cells while control cells transfected with the empty vector still underwent apoptosis (Figure 3). These results suggest that cytotoxic drugs sensitize cells for CD95-mediated apoptosis.

Drug-induced CD95 expression

Drug-induced sensitivity to CD95-mediated apoptosis may be due to an increased expression of CD95. We therefore treated Nalm6 cells with doxorubicin and measured CD95 expression by RT-PCR. Dose dependent (0.01–0.1 μg/ml) upregulation of

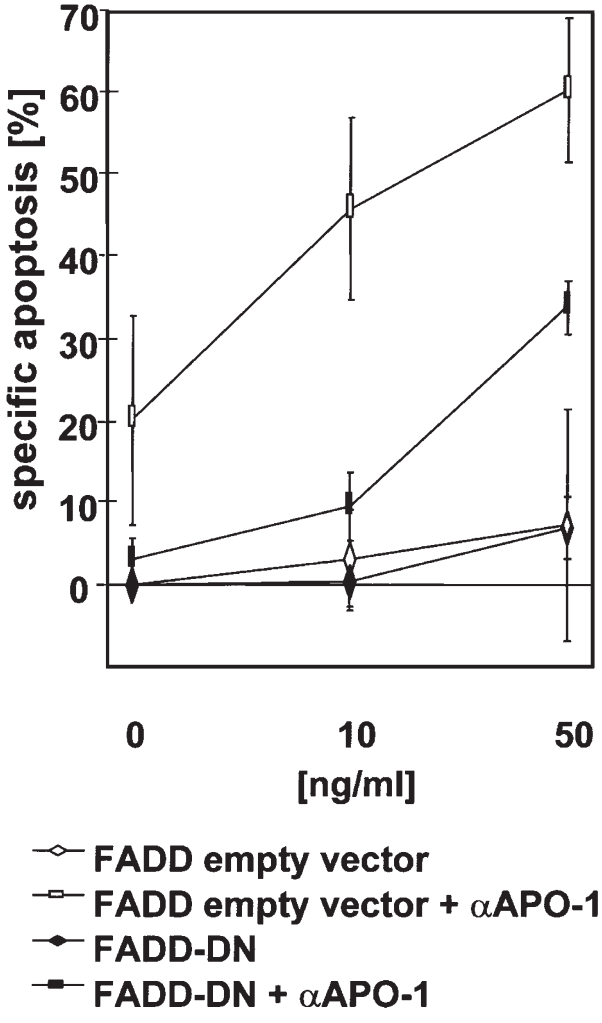

Figure 3 Anti-APO-1 induced apoptosis in sensitized Nalm6 cells is blocked in FADD-DN transient transfected cells. Cells were transfected with a FADD-DN expression construct or empty vector in the control together with a GFP-expression construct. Following transfection the cells were left either untreated or incubated with doxorubicin (0.01 μg/ml) for 12 h. After washing the cells were left either untreated or incubated with anti-APO-1 for 24 h. Apoptosis of transfected cells, which were defined by the green fluorescent protein (GFP), was determined by flow cytometry (FSC/SSC) and specific apoptosis was calculated as described in Materials and methods.

CD95 mRNA was found 4 to 12 h after doxorubicin treatment with a maximal increase between 8 to 12 h (Figure 4a, data not shown). These data were confirmed by flow cytometry showing upregulation of CD95 protein 20 h after treatment of Nalm6 cells (Figure 4b). However, the ability to induce CD95 varied depending on the drugs and cell lines used (Figure 4c).

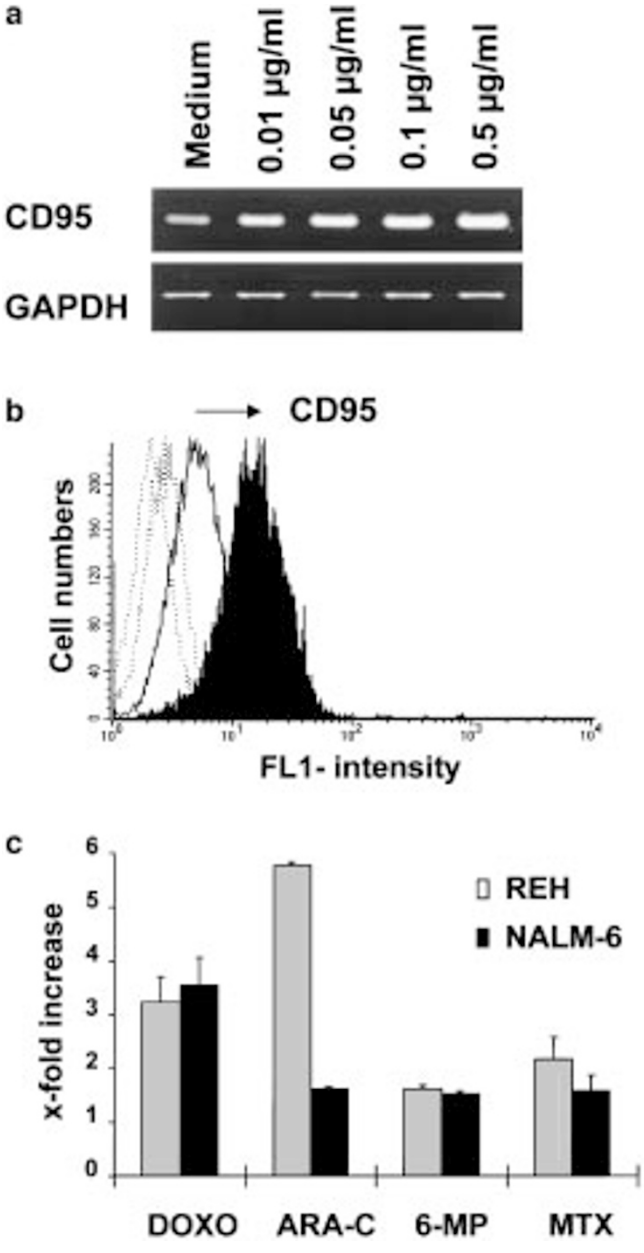

Figure 4 Upregulation of the CD95 receptor after drug treatment in Nalm6 and REH. (a) mRNA expression of CD95 in Nalm6 using different concentrations of doxorubicin was examined by RT-PCR 9 h after drug treatment. (b) FACScan analysis of CD95 surface expression in untreated Nalm6 cells (white curve) or the same cells incubated with 0.05 µg/ml doxorubicin for 20 h (filled curve). IgG1 antibodies were used as a control (dots). (c) Induction of CD95 surface protein by incubation of Nalm6 and REH cells with the drugs was measured by flow cytometry. Nalm6 and REH were treated 24 h with 0.05 µg/ml doxorubicin or 0.1 µg/ml Ara-C and 48 h with µg/ml 6-MP and 0.5 µg/ml MTX. X-fold increase was calculated as mean fluorescence intensity (MFI) of treated cells divided by MFI of untreated cells.

Low level caspase activation is not sufficient to trigger apoptosis

We further analyzed downstream effector molecules in death signaling pathways such as caspases, which are involved in drug- and CD95-mediated apoptosis.[39,40] Nalm6 cells were treated with anti-APO-1 antibody and cleavage of the CD95 receptor-proximal caspase FLICE (caspase 8) and the downstream caspase CPP32 (caspase 3) was monitored by Western blot analysis. Increased FLICE cleavage into the p18 active subunit was observed in cells pre-treated with doxorubicin and subsequently exposed to anti-APO-1 (Figure 5). In addition, CPP32 was proteolytically processed and PARP, one of the known substrates for CPP32,[41] was cleaved to its M_T 85 000 fragment. Interestingly, anti-APO-1 antibody alone also induced a low level of CPP32 activation.

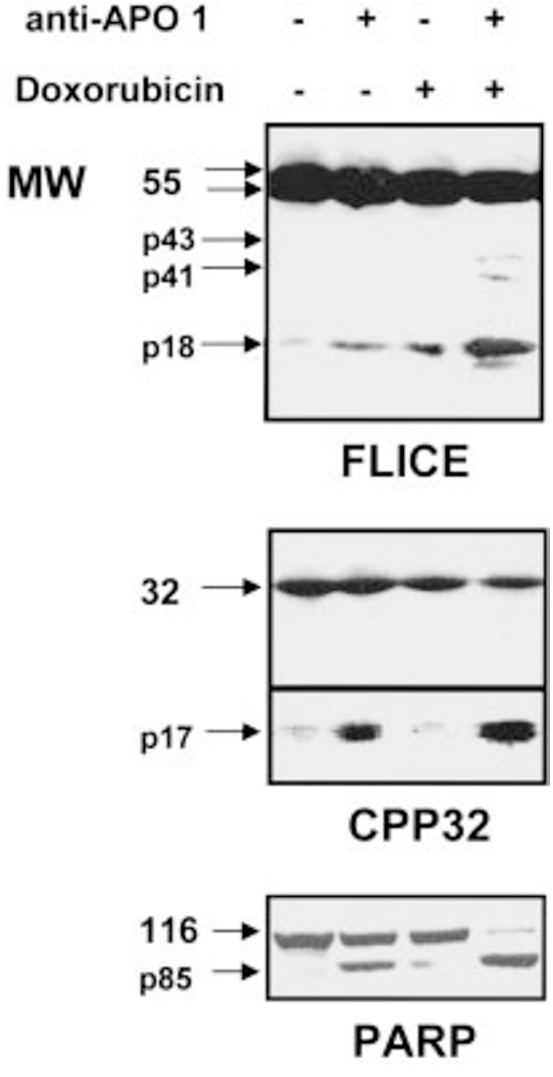

Figure 5 Caspase activation alone is not sufficient to trigger apoptosis. Nalm6 cells were cultured in medium or 0.01 µg/ml doxorubicin. Following washing they were incubated with 1 µg/ml anti-APO-1 and 10 ng/ml protein A or left untreated. 24 h later protein was isolated and FLICE, CPP32 and PARP cleavage was examined by Western blot.

Doxorubicin induces disturbance of mitochondrial function

Mitochondria have recently been implicated in the control of apoptosis, including chemotherapy and CD95 signaling.[42–44] In particular in type II cells mitochondria appear to be a crucial amplifier of the CD95 death signal.[45] We therefore investigated the effect of doxorubicin on mitochondrial function. Treatment of Nalm6 cells with doxorubicin (0.01–0.05 μg/ml) caused $\Delta\psi_m$ disruption followed by hyperproduction of ROS (data not shown). Both events have been shown to contribute to apoptosis,[42] however at the concentrations used we did not observe a significant amount of cell death after 12 h of drug exposure. After the 24 h drug-free interval all cells were viable and displayed normal features of mitochondrial function. Collapse of $\Delta\psi_m$ and ROS generation was only detected when Nalm6 cells were pre-treated with doxorubicin prior to triggering with anti-APO-1 antibody (Figure 6). While doxorubicin is able to induce $\Delta\psi_m$ and ROS but not cell death, anti-APO-1 treatment induces both $\Delta\psi_m$/ROS and apoptosis.

Involvement of Bcl-2 family proteins in sensitization for CD95-induced apoptosis

Bcl-2 and Bcl-x_L have been shown to block cell death by preventing mitochondrial permeability transition and subsequent breakdown of $\Delta\psi_m$.[43] To examine the role of Bcl-2 and Bcl-x_L in controlling anti-APO-1 sensitivity we incubated untreated cells and cells pre-treated with doxorubicin with or without anti-APO-1 for 24 h. Pre-treatment with doxorubicin caused a decrease in Bcl-x_L expression, but Bcl-2 expression was unaffected (data not shown); the pro-apoptotic protein Bcl-x_S was further induced by anti-APO-1 (Figure 7).

Drugs sensitize for LAK cell kill

Besides direct cytotoxic effects, anticancer drugs may render tumor cells sensitive for mechanisms of immune surveillance. Therefore, we co-incubated IL-2-activated human effector lymphocytes (LAK cells), which are known to be highly cytotoxic against human leukemic cells with Nalm6 or REH at

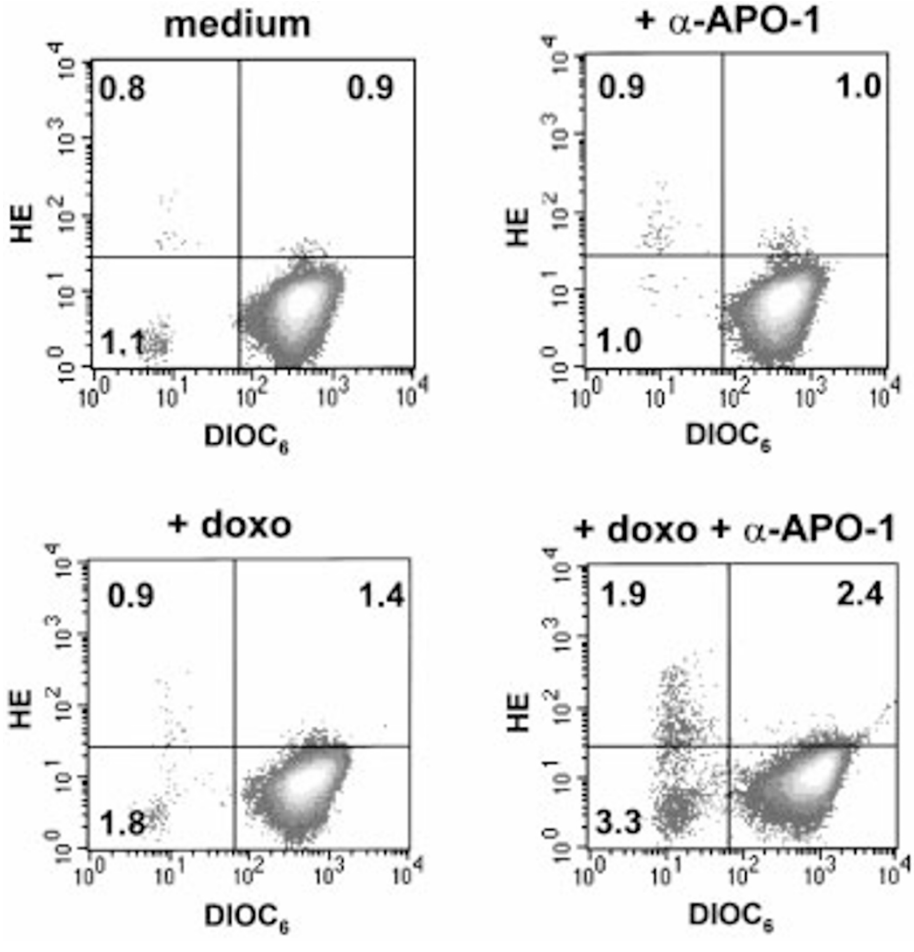

Figure 6 Disturbance of mitochondrial transmembrane potential and generation of ROS after doxorubicin and anti-APO-1 treatment. Nalm6 cells were either left untreated or incubated 12 h with 0.01 μg/ml doxorubicin. After washing cells were divided and left either untreated or incubated for 24 h with anti-APO-1. Loss of mitochondrial transmembrane potential ($DIOC_6(3)$) and generation of ROS (HE) was detected as described in Materials and methods by flow cytometry gating on the viable cells via their FSC/SSC profile.

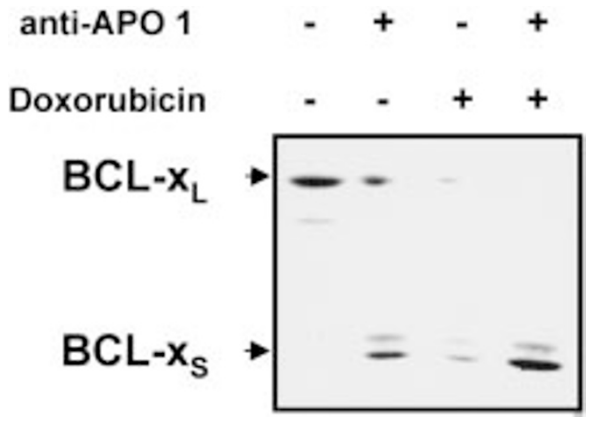

Figure 7 Downregulation of Bcl-x_L after doxorubicin treatment. Nalm6 cells were left untreated or stimulated with 0.01 μg/ml doxorubicin. After washing cells were incubated with 1 μg/ml anti-APO-1 and 10 ng/ml protein A or left untreated. 24 h later proteins were isolated and Western blot analysis of Bcl-x expression was performed.

different effector to target cell ratios. However, LAK cells failed to kill Nalm6 and REH target cells efficiently (Figure 8 and data not shown). LAK cells have been shown to mediate their cytolytic activity by perforin, CD95-L and TNFα.[23,24] Preincubation of target cells with doxorubicin, cytarabine, 6-mercaptopurine or methotrexate strongly increased LAK-induced cell death (Figure 8). These data suggest that anticancer drugs may indeed augment the efficiency of the immune system in elimination of precursor B-lineage ALL cells during chemotherapy by increasing the sensitivity of these cells for apoptosis-inducing stimuli.

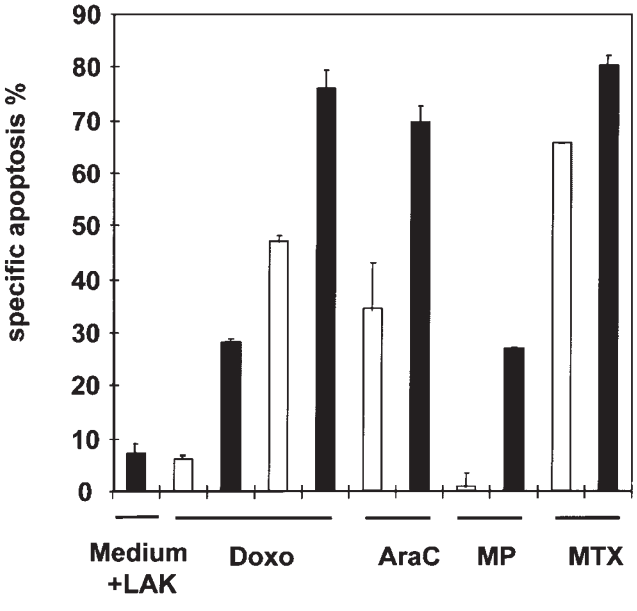

Figure 8 Cytotoxic drugs sensitize for LAK induced cell death. 5×10^5 Nalm6 target cells were left untreated or incubated with 0.01 μg/ml doxorubicin, 0.01 μg/ml arabinoside, 0.1 μg/ml 6-mercaptopurine or 0.5 μg/ml methotrexate for 12 h at 37°C with 5% CO_2. After washing they were cultured in RPMI and seeded in 96-well plates. Finally, 10 day IL-2 stimulated LAK cells were added to one half (killer to target ratio 1:4) and co-incubated for 24 h. Apoptosis was measured by FACS annexin V analysis of gated CD19-positive target cells incubated with (black bars) or without (white bars) LAK cells.

Discussion

The CD95 system appears to be a major effector pathway by which anticancer drugs, as well as cytotoxic T cells mediate apoptosis.[5,6,46,47] Expression of CD95 and CD95-L is induced by cytotoxic drugs in different tumor cell lines[5,19,28,48] and interaction of both molecules leads to apoptosis.[49] However, CD95-independent apoptosis induced by anticancer drugs has also been described. Here, we investigated the role of the CD95 system during anticancer drug treatment of human B-ALL cells *in vitro*. Similar to clinical strategies we used a short pulse of drug exposure at levels usually seen *in vivo* followed by a drug-free incubation period *in vitro*. This experimental setting revealed a steep increase in the sensitivity of precursor B-ALL cells constitutively resistant towards CD95 and LAK cell-induced apoptosis. Combination treatment of cytotoxic drugs, as well as certain cytokines with CD95 agonistic antibodies, has also been demonstrated to increase apoptosis in tumor cells.[27–29,50] This indicates that the CD95 signaling pathway may be reversibly modulated during tumor progression. Correspondingly, we found that the CD95-resistant precursor B-ALL cell lines used in our study expressed low basal levels of CD95 compared to anti-CD95-sensitive cells. CD95 mRNA and protein expression in these cells was upregulated following pulse treatment with cytotoxic drugs. In contrast to T-ALL cell lines CD95-L mRNA and protein was not inducible in these B-ALL cell lines (unpublished data and Ref. 34). Therefore we hypothesized that cytotoxic drugs may sensitize these cell lines towards physiological apoptotic signals. Indeed exogenous triggering of CD95 by agonistic antibodies induced apoptosis only in pulse-treated, but not in untreated cells. Consistent with a function of the CD95 system in mediating apoptosis in drug-sensitized previously anti-CD95 resistant cells, anti-APO-1 F(ab')$_2$ antibody fragments (unpublished data) or a dominant-negative FADD-construct inhibited cell death induced by anti-APO-1 triggering under these circumstances.

Surprisingly, upregulation of the CD95 receptor did not correlate with the degree of sensitivity induced by different cytotoxic drugs towards anti-APO-1-mediated cell death. Thus, additional mechanisms are responsible for the anti-CD95-resistant phenotype. Downregulation of anti-apoptotic proteins such as Bcl-2 or Bcl-x_L, as well as upregulation of pro-apoptotic factors such as Bax, AIF or cytochrome c may contribute to apoptosis sensitivity.[42,51,52] In our experiments doxorubicin treatment was followed by decreased Bcl-x_L protein expression, while Bcl-x_L mRNA as well as Bcl-2 and Bax protein expression was not affected (unpublished data). Overexpression of Bcl-x_L has been shown to antagonize mitochondrial dysfunction induced by cytotoxic drugs.[43] The loss of $\Delta\psi_m$ and an increased ROS production by mitochondria usually precede apoptotic cell death. However, since we found drug-induced distruption of $\Delta\psi_m$ and generation of ROS in the absence of significant apoptosis during the first 12 h of doxorubicin treatment in anti-CD95 resistant cells (unpublished data), this may not be an irreversible event. Indeed, mitochondrial pertubations disappeared after washing cells and subsequent *in vitro* culture in drug-free medium. Resistance to drug-induced disruption of the mitochondrial membrane potential was only found in doxorubicin super-resistant cells which have been selected from parental cells by continuous culture in doxorubicin (unpublished data).

Interestingly, the changes in mitochondrial function are paralleled by activation and cleavage of caspases, an event that usually occurs downstream of mitochondria and is induced

by the release of mitochondrial-derived apoptosis-inducing factors.[44,52] The fact that drug treatment, as well as CD95 triggering of B-lineage ALL cells, did induce low level activation of caspases in the absence of significant cell death indicates that intracellular repair mechanisms may be able to compensate for a certain level of pro-apoptotic signaling. Only if drug-exposed pre-sensitized cells are restimulated via the CD95 receptor or by LAK cells, the cell death program fully proceeds.

Taken together, mechanisms which increase the sensitivity of previously resistant cells include both the upregulation of the CD95 receptor (which increases the intensity of the pro-apoptotic second signal), as well as a decreased capacity of the cell to cope with mitochondrial perturbation probably due to an increased function of the pro-apoptotic Bcl-x_S protein. These data might have clinical relevance since they explain the effectiveness of low-dose 'maintenance' chemotherapy in certain tumors. Furthermore, drug treatment *in vivo* may activate T cells which in turn produce cytokines like CD95-L, thereby killing sensitized tumor target cells. This will define a new role for the immune system in interacting with sensitive and resistant malignant cells during chemotherapy.

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