Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand–Mediated Proliferation of Tumor Cells with Receptor-Proximal Apoptosis Defects

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Abstract

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) might represent a future cytotoxic drug to treat cancer as it induces apoptosis in tumor cells without toxicity in animal trials. We recently described that in contrast to apoptosis, TRAIL mediates tumor cell survival and proliferation in certain tumor cells. Here we studied the effect of TRAIL on 18 cell lines and 53 primary leukemia cells and classified these tumor cells into four groups: TRAIL, anti-DR4 or anti-DR5 induced apoptosis in group A cells, whereas they had no effect on group 0 cells and mediated proliferation in group P cells. To our surprise, TRAIL induced simultaneous apoptosis and proliferation in group AP cells. More than 20% of all cells tested belonged to group P and showed TRAIL-mediated proliferation even in the presence of certain cytotoxic drugs but not inhibitors of nuclear factor-κB. Transfection with B-cell leukemia/lymphoma protein 2 transformed group A cells into group 0 cells, whereas transfection with Fas-associated polypeptide with death domain (FADD)–like interleukin-1-converting enzyme–inhibitory protein (FLIP) transformed them into group AP cells. Loss of caspase-8 or transfection of dominant-negative FADD transformed group A cells into group P cells. Taken together, our data suggest that proliferation is a frequent effect of TRAIL on tumor cells, which is related to receptor-proximal apoptosis defects at the level of the death-inducing signaling complex and should be prevented during antitumor therapy with TRAIL. (Cancer Res 2005; 65(17): 7888-95)

Introduction

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) is a member of the TNF family, which effectively induces apoptosis in a variety of tumor cells of different origin (1), especially in the presence of cytotoxic drugs (2). As TRAIL seems nontoxic to benign human cells and in animal trials (3), TRAIL or antibodies directed against the apoptosis inducing TRAIL receptors DR4 or DR5 might represent future cytotoxic drugs to treat cancer. For intracellular apoptosis signal transduction, the adapter molecule Fas-associated polypeptide with death domain (FADD) binds to the multimerized TRAIL receptor attracting caspase-8 (cysteine aspartyl-specific protease-8 or FADD-like interleukin-1-converting enzyme, FLICE) to form the death-inducing signaling complex (DISC; ref. 4). In competition to caspase-8, FLICE-inhibitory protein (FLIP) binds to FADD and inhibits apoptosis induction by TRAIL. Apart from inhibition of apoptosis, FLIP contains additional apoptotic and antiapoptotic functions including activation of the transcription factor nuclear factor-κB (NF-κB; ref. 5). Activated caspase-8 either directly activates downstream caspases in type I cells or activates mitochondria in type II cells like JURKAT cells (6) which can be inhibited by B-cell leukemia/lymphoma protein 2 (Bcl-2). Apoptosis induction by TRAIL is mostly dependent on caspase activation and is disabled by inhibitors like the broad spectrum caspase inhibitor zVAD-fmk. In addition to induction of apoptosis, TRAIL contains various antiapoptotic functions on normal cells (7) and activates NF-κB even in tumor cells which attenuates apoptosis induction by TRAIL (8, 9). We recently showed that via NF-κB TRAIL mediates survival and proliferation of certain tumor cells resistant against TRAIL-induced apoptosis (10). To study this new characteristic of TRAIL in more detail, we examined 18 cell lines and 53 primary leukemia cells for the effect of TRAIL.

Materials and Methods

Materials. Four different preparations of TRAIL were used giving similar results: TRAIL without his-tag produced in *Escherichia coli* (Peprotech, Tebu-Bio, Offenbach, Germany; this form of TRAIL was used exclusively in primary leukemia cells), with his-tag produced in *Pichia pastoris* (11), with his-tag produced in *E. coli* (Biomol, Hamburg, Germany) and with his-tag produced in *E. coli* and multimerized (Alexis, Grünberg, Germany; this form of TRAIL was used in most experiments with cell lines). Antibodies were anti-Bcl-2 from Santa Cruz Biotechnology (Heidelberg, Germany); anti-FADD from BD Biosciences (Heidelberg, Germany); anti-α-tubulin from Merck Biosciences (Schwalbach, Germany) and anti-FLIP (recognizing both FLIP long and FLIP short), anti-caspase-8, anti-DR4, anti-DR5, and TRAIL-R2-Fc from Alexis (Grünberg, Germany). CFSE-DA was obtained from Molecular Probes (Karlsruhe, Germany); Dacarbacin from Sigma (Deisenhofen, Germany); U0126, SP600125, Wortmannin, SN50, and 6-amino-4-(4-phenoxyphenylethylamino) quinazoline from Calbiochem (Schwalbach, Germany). All further agents were supplied by Sigma.

Cell culture. All cell lines were cultured in RPMI 1640 (Life Technologies, Karlsruhe, Germany) supplemented with 10% FCS (Seromed, Berlin, Germany) and 2 mmol/L glutamine (Life Technologies). For primary leukemia cells, FCS was used at 20%. The following derivative cell lines were used: cell lines overexpressing FADD-DN described by Fulda et al. (12), Bcl-2 overexpressing cells by Armstrong et al. (13), and SHEP cells overexpressing FLIP long generated by Beltinger et al.[4] Lack of caspase-8 in

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-04-4278

[4] Unpublished data.

KELLY and Sy5y neuroblastoma cells was first described by Fulda et al. (14), in J-TR cells and CEM-TR cells by Ehrhardt et al. (10), and in JURKAT Casp8$^{-/-}$ by Juo et al. (15). MCF-7 cells were characterized by Medema et al. (16).

Primary leukemic blasts were obtained from 53 children treated for acute leukemia at the Ludwig Maximilians University's children's hospitals during 2002 to 2004. Samples were obtained by bone marrow puncture before onset of therapy, were isolated using Ficoll Isopaque (Amersham, Uppsala, Sweden), and were stimulated directly after isolation.

Measurement of tumor cell proliferation and apoptosis. To measure cell concentration with high sensitivity, we established a new test called *Concetest* that was done as follows: cells of interest were harvested using trypsin (Life Technologies) and were kept on a shaker for homogeneous suspension. Calibrator cells were diluted to exactly 0.2×10^6 cells/mL as counted using Neugebauer cell chamber, were stained using CFDA-SE (0.25 μmol/L; without washing to prevent alteration in cell concentration; CFSE still present in the media explains the shift of uncolored cells detectable in Fig. 1*A*), and were mixed together with cells of interest directly before analysis in quadruplicates as a duplicate as each a 1:1 and a 1:2 mixture. Percentage of living unstained cells was measured by FACScan (Becton Dickinson, Heidelberg, Germany) in fluorescence-1, variation between quadruplicates of <10% was accepted. Concentration of cells of interest were calculated as [percentage of uncolored cells / (100 − percentage of uncolored cells) × concentration of colored cells]. To measure cell adhesion after seeding, "start" cells were harvested at the time point of stimulation and kept at 4°C in the presence of 4% paraformaldehyde until end of the experiment where all samples were measured at the same time.

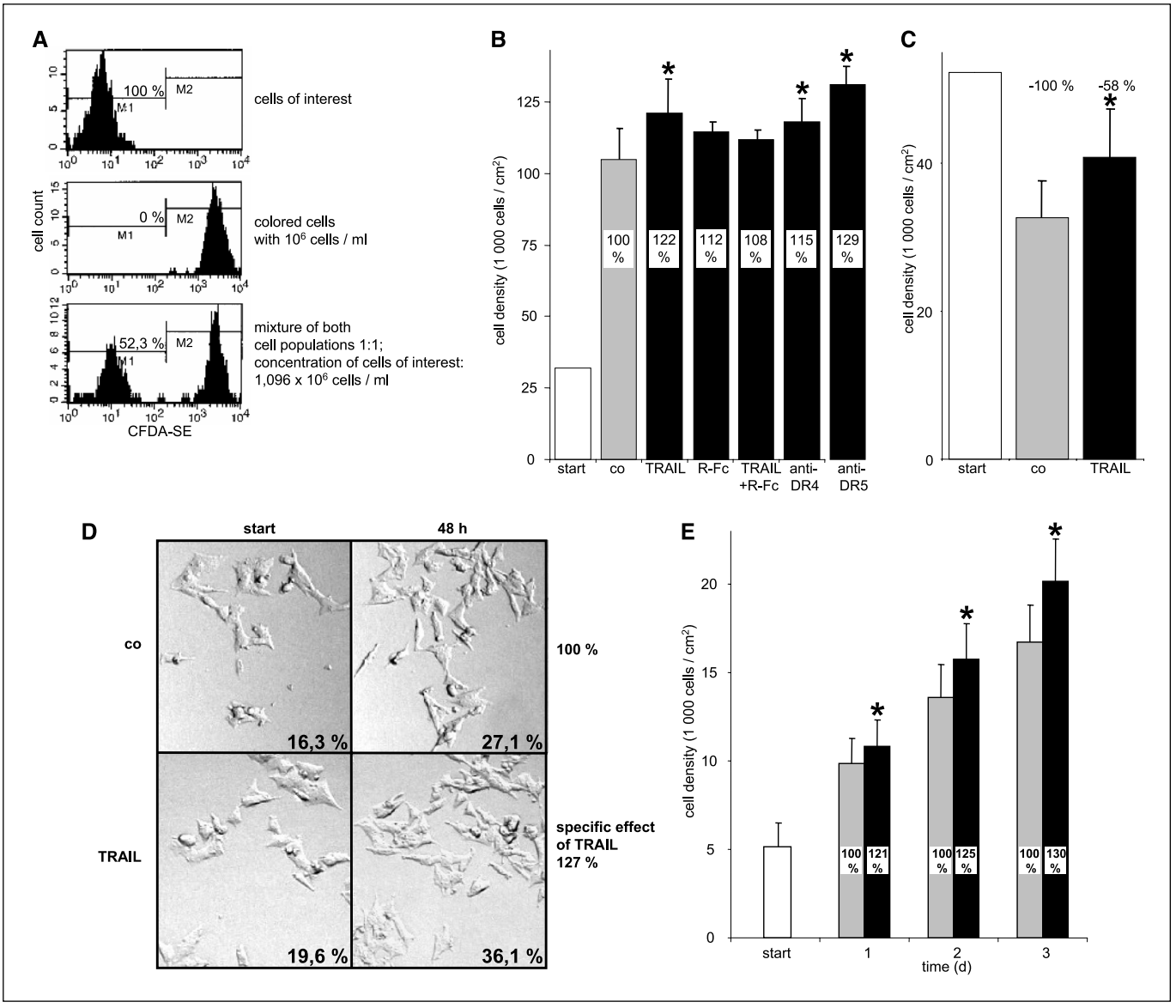

Figure 1. TRAIL-mediated proliferation in KELLY neuroblastoma cells. *A,* Concetest was established, a new technique to measure cell concentration: uncolored cells of interest with unknown concentration were mixed together with CFSE-DA colored cells with known concentration. Percentage of uncolored cells were measured in fluorescence-1 using FACScan and cell concentration was calculated thereof. *B-E,* KELLY neuroblastoma cells were incubated with 40 ng/mL TRAIL and Concetest was done after 48 hours. *B,* cells were kept under normal culture conditions for spontaneous growth of control cells; anti-DR4 and anti-DR 5 were both used at 1 μg/mL; TRAIL-R2-Fc (R-Fc) was used at 10 μg/mL. *C,* cells were kept in PBS for spontaneous death of control cells. *D-E,* cell density was measured using cellscreen. *D,* typical light microscopic pictures after 48 hours of stimulation; percentage within image indicates % area covered with cells. *E,* kinetic at FCS 10%. *Columns,* means of at least three independent experiments, numbers within or above columns indicate specific effect of TRAIL compared with control; *bars,* ±SE (*B*, *C*, and *E*; see Materials and Methods for details). *, $P < 0.05$, compared with control using one-way ANOVA or paired *t* test.

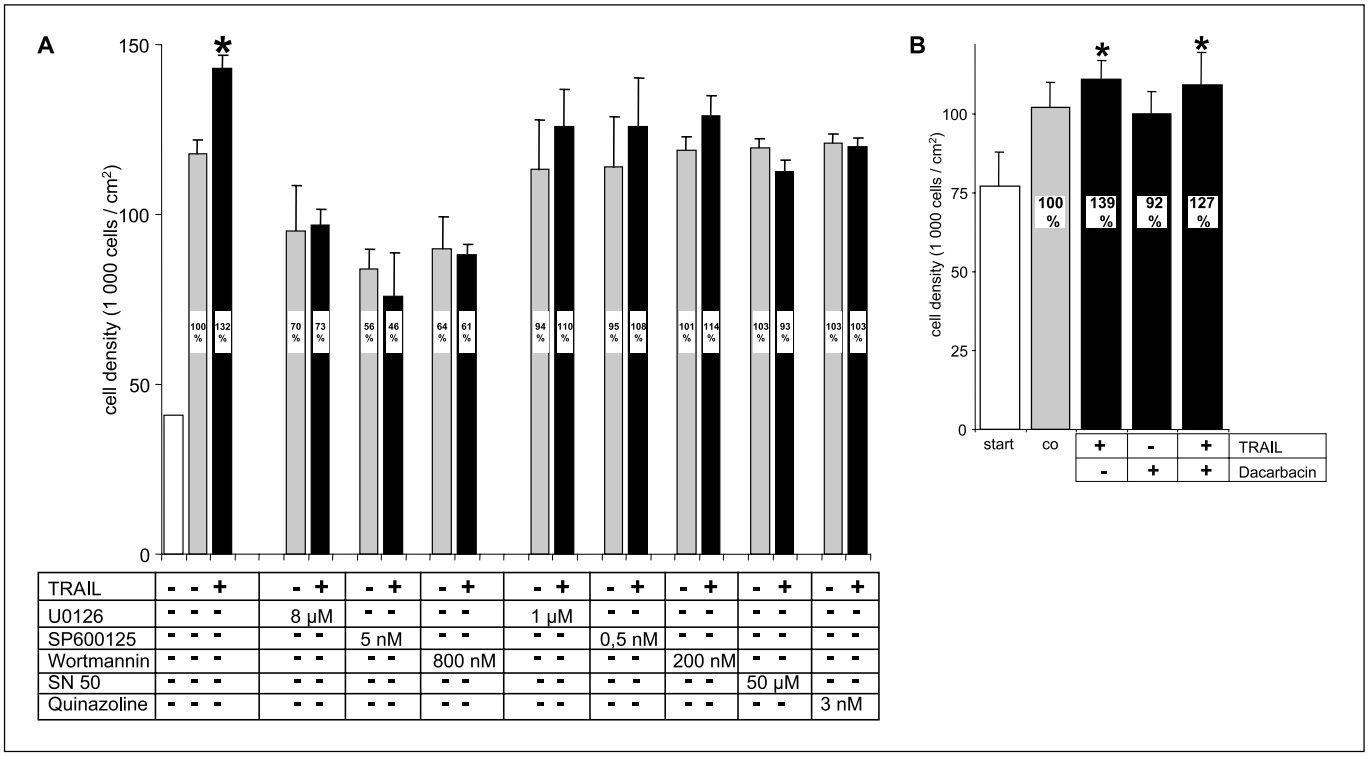

Figure 2. TRAIL-mediated proliferation in the presence of cytotoxic drugs and growth inhibitors. *A,* SHEP-FLIP neuroblastoma cells were treated with growth inhibitors for 30 minutes followed by addition of 40 ng/mL TRAIL for another 24 hours as indicated in three independent experiments. *Quinazoline,* 6-amino-4-(4-phenoxyphenylethylamino) quinazoline. *B,* KELLY neuroblastoma cells were incubated with 40 ng/mL TRAIL and/or 27 μmol/L dacarbacin (5 μg/mL) as indicated for 72 hours. Cell concentration was measured using Concetest and is depicted as in Fig. 1.

The *CellTiter-Glo* Cell Viability Assay (Promega, Mannheim, Germany) was used according to the manufacturers instructions to measure cellular ATP content.

The *cellscreen* (Innovatis AG, Bielefeld, Germany) was used according to the manufacturer's instructions to take pictures of exactly the same cells in culture over time. Percentage of area covered with cells was estimated by automated analysis of each picture using the PA adhesion software (Innovatis) and cell concentration was calculated thereof using a standard curve.

In primary samples, all visible *colonies* were counted in light microscopy by a blinded operator.

Apoptosis was measured by DNA fragmentation in FACScan (17) or by forwardside scatter analysis for primary leukemia cells. For *mitochondrial membrane potential*, cells were stained with $DiOC_6$ (Sigma; 0.45 μg/mL) for 15 minutes at 37°C and measured in fluorescence-1 in FACScan.

Calculation of tumor necrosis factor–related apoptosis-inducing ligand–specific effect. In Concetest and cellscreen measurements, specific growth by TRAIL was calculated as [(concentration in the presence of TRAIL − concentration at start of the experiment) / (concentration under control conditions − concentration at start of the experiment) × 100].

Specific apoptosis as well as specific loss of mitochondrial membrane potential was calculated as [(absolute apoptosis of TRAIL-treated cells − absolute apoptosis of untreated cells) / (100 − absolute apoptosis of untreated cells) × 100]. Specific viability was calculated as 100 − specific apoptosis.

In colony formation in primary cells as well as in Cell Titer Glow Viability assay, TRAIL-specific effect was calculated as [(number of colonies / absorbance in the presence of TRAIL) / (number of colonies / absorbance in the absence of TRAIL) × 100].

Quantitative real-time PCR, Western blot, and electrophoretic mobility shift assay. Total RNA was extracted using the High Pure RNA Isolation kit (Roche, Grenzach, Germany). After reverse transcription, cDNA was diluted 1:100 and 1:1,000 and was subjected to real-time PCR using IQ SYBR Green Supermix (Bio-Rad, Munich, Germany) and the following two primer pairs: *flip* forward 5′-tggaccttgtggttgagttg-3′, *flip* reverse 5′-ttggattgctgcttggaga-3′ (amplifying both *flip* long and *flip* short; ref. 18) and the housekeeping gene *hrpt* forward 5′-ggtggagatgatctctcaactttaa-3′, *hrpt* reverse 5′-aggaaagcaaagtctgcattgtt-3′ (19) both from Metabion (Munich, Germany). Numbers of cycles necessary to cross the detection threshold (cT) of *hrpt* was subtracted from *flip* (ΔcT), mean was calculated of both concentrations and subtracted from mean obtained for BOE calibrator cells (ΔΔcT). To obtain the result as fold difference, mean of the three independent experiments was calculated as $2^{-\Delta\Delta cT}$.

Western blot and electrophoretic mobility shift assay were done as described previously (10).

Statistical analysis and presentation of data. Whenever possible, paired *t* test or one-way repeated measure ANOVA was used. Student-Newman-Keuls *t* tests were applied to isolate statistical differences whenever a difference was detected by ANOVA. *P*s < 0.05 were considered significant. In all figures, data were presented as mean of at least three independent experiments ± SE, if SE was >5%; SE <5% was not depicted.

Results

We recently described that TRAIL mediates cell survival and cell growth in certain tumor cells. To study this new characteristic of TRAIL in more detail, we established a new FACScan-based technique called Concetest to measure cell concentration. In Concetest, cells of interest were mixed together with stained calibrator cells of known concentration, percentage of living unstained cells was measured by FACScan and concentration of cells of interest was calculated thereof (Fig. 1*A*; for detailed description, see Materials and Methods). This technique is simple and cheap but requires single cell suspension and exact pipetting. Using Concetest, KELLY neuroblastoma cells showed significantly increased tumor cell growth upon stimulation with TRAIL independently of the presence of his-tag or multimerization of

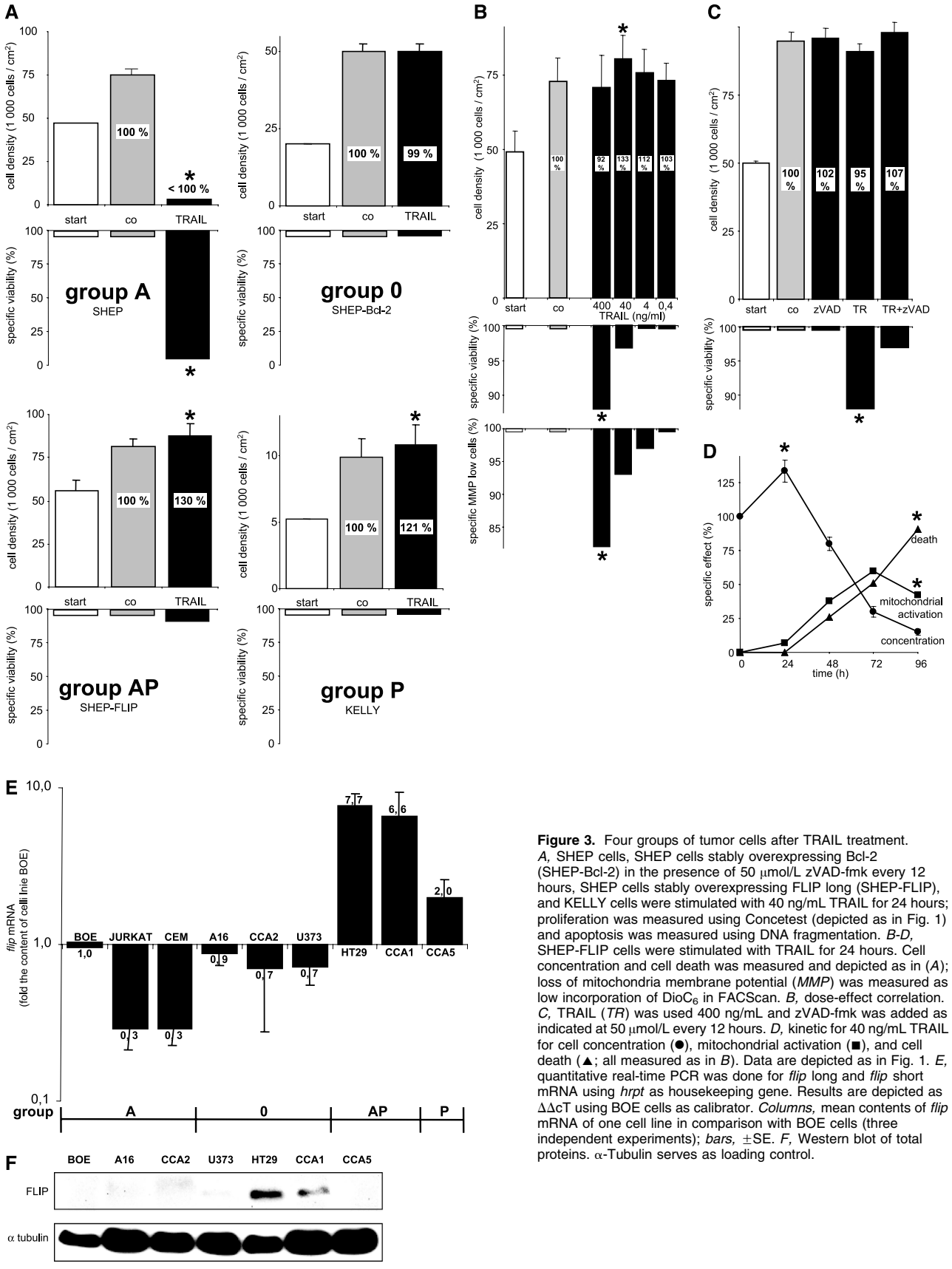

Figure 3. Four groups of tumor cells after TRAIL treatment. *A,* SHEP cells, SHEP cells stably overexpressing Bcl-2 (SHEP-Bcl-2) in the presence of 50 μmol/L zVAD-fmk every 12 hours, SHEP cells stably overexpressing FLIP long (SHEP-FLIP), and KELLY cells were stimulated with 40 ng/mL TRAIL for 24 hours; proliferation was measured using Concetest (depicted as in Fig. 1) and apoptosis was measured using DNA fragmentation. *B-D,* SHEP-FLIP cells were stimulated with TRAIL for 24 hours. Cell concentration and cell death was measured and depicted as in (*A*); loss of mitochondria membrane potential (*MMP*) was measured as low incorporation of $DioC_6$ in FACScan. *B,* dose-effect correlation. *C,* TRAIL (*TR*) was used 400 ng/mL and zVAD-fmk was added as indicated at 50 μmol/L every 12 hours. *D,* kinetic for 40 ng/mL TRAIL for cell concentration (●), mitochondrial activation (■), and cell death (▲; all measured as in *B*). Data are depicted as in Fig. 1. *E,* quantitative real-time PCR was done for *flip* long and *flip* short mRNA using *hrpt* as housekeeping gene. Results are depicted as ΔΔcT using BOE cells as calibrator. *Columns,* mean contents of *flip* mRNA of one cell line in comparison with BOE cells (three independent experiments); *bars,* ±SE. *F,* Western blot of total proteins. α-Tubulin serves as loading control.

Table 1. Effect of TRAIL on 18 tumor cell lines

Group	Cell line	Origin	Apoptosis defect
A	A20	Mouse lymphoma	—
	BOE	B-cell leukemia	—
	U87MG	Glioblastoma	—
	Among many others		
0	MCF-7	Breast carcinoma	Loss of caspase-3, overexpression of Bcl-xL
	A16	Renal cell carcinoma	Unknown
	CCA2	Renal cell carcinoma	Unknown
	U373	Glioblastoma	Unknown
AP	CCA1	Renal cell carcinoma	Unknown
	HT29	Colon carcinoma	Unknown
P	KELLY	Neuroblastoma	Loss of caspase-8
	Sy5y	Neuroblastoma	Loss of caspase-8
	CCA5	Renal cell carcinoma	Unknown
	MPC11	Mouse lymphoma	Unknown

NOTE: Cells were stimulated with TRAIL 40 ng/mL for 24 hours in at least five independent experiments. Cell death was measured using DNA fragmentation. Cell proliferation was measured using Concetest, cell screen, and ATP content in parallel. Cells of group A or AP showed in mean at least 10% specific apoptosis, cells of group P or AP showed in mean at least 120% specific proliferation by TRAIL, which was statistically significant over control growth.

TRAIL (data not shown) that was inhibited by soluble TRAIL receptor to background levels (Fig. 1*B*). For yet unknown reasons, TRAIL-R2-Fc slightly elevated proliferation. Furthermore, antibodies directed against either DR4 or DR5 induced proliferation of tumor cells (Fig. 1*B*) extending the proliferative effect to different TRAIL receptor agonists. TRAIL-mediated proliferation was accompanied by increase in percentage of Ki67-positive cells (data not shown). Under cell culture conditions where cells died spontaneously, TRAIL inhibited cell death and increased cell survival (Fig. 1*C*). TRAIL-mediated proliferation and survival of KELLY cells were also seen at very low concentrations of TRAIL (0.4 ng/mL; data not shown). In a different technical approach, daily photographs of tumor cells were taken using cellscreen (Fig. 1*D*) and cell concentration was estimated as percentage of area covered with cells. In contrast to suspension cells, spontaneous cell growth of adherent cells in culture was linear and not exponential (Fig. 1*E*). TRAIL accelerated tumor cell proliferation continuously over several days (Fig. 1*E*), which led to a significant decrease in doubling time from 24 to 19 hours and a doubling in tumor cell count by TRAIL within about 6 days. TRAIL-mediated tumor cell proliferation was further verified measuring cellular ATP content (Supplementary Fig. S1).

Inhibition of tumor necrosis factor–related apoptosis-inducing ligand–mediated tumor cell proliferation. TRAIL-mediated proliferation was significantly reduced by suboptimal cell culture conditions or serum starvation (data not shown). This may indicate a requirement for intact survival signaling (e.g., by NF-κB), which was constitutively activated at low levels in all cell lines used in our experiments (data not shown). TRAIL-mediated proliferation was further blocked, when spontaneous growth was suppressed by growth inhibitors of different survival and proliferation pathways like mitogen-activated protein kinases (U0126 inhibits mitogen-activated protein kinase kinase-1, c-*jun*-NH_2-kinase kinases (SP600125), phosphatidylinositol 3-kinase, and thereby protein kinase B/Akt (Wortmannin), or activation of NF-κB [SN50 or 6-amino-4-(4-phenoxyphenylethylamino)quinazoline]. In contrast, when growth inhibitors were added at concentrations were spontaneous growth was unaffected, only inhibitors of NF-κB were able to completely block TRAIL-mediated proliferation suggesting a prominent role of both constitutive and TRAIL-mediated activation of NF-κB for TRAIL-mediated proliferation of tumor cells.

Important for potential future clinical use, all cytotoxic drugs which induce relevant (>10% specific) apoptosis in target cells were able to inhibit TRAIL-mediated proliferation (data not shown). In contrast, if tumor cells showed complete resistance against apoptosis induction by a certain cytotoxic drug at peak plasma concentration, TRAIL mediated proliferation in the presence of this drug (e.g., dacarbacin; Fig. 2*B*) or dexamethasone (data not shown) on KELLY cells or doxorubicin on J-TR cells (data not shown).

Four groups of tumor cells after tumor necrosis factor–related apoptosis-inducing ligand treatment. Various tumor cell lines of different origin were tested for the effect of TRAIL. Apoptosis induction by TRAIL was considered relevant at >10% specific apoptosis and proliferation by TRAIL at >120% specific cell growth. As expected, TRAIL induced relevant apoptosis within 24 hours in many cell lines that were called group A cell lines (Fig. 3*A*). TRAIL had no relevant effect on tumor cell lines of group 0, whereas in some of these cell lines TRAIL induced apoptosis at later time points (e.g., in MCF-7 cells). TRAIL mediated survival or proliferation in group P cell lines.

To our surprise, we found cell lines in which TRAIL induced both apoptosis and proliferation and which were called group AP cell lines. Apoptosis by TRAIL in AP group cells was accompanied by loss of mitochondrial membrane potential and formation of sub-G_1 DNA fragments (Fig. 3*B*). Whether TRAIL induced apoptosis or proliferation in group AP cell lines was determined (a) by the concentration of TRAIL used with high concentrations inducing apoptosis and low concentrations inducing proliferation (Fig. 3*B*); (b) by the activation of caspases as the broad spectrum caspase inhibitor zVAD-fmk was able to inhibit apoptosis induction by TRAIL and to maintain TRAIL-mediated proliferation over time (Fig. 3*C*); (c) by the incubation time, as proliferation was

overturned by apoptosis at later time points for all concentrations of TRAIL used (Fig. 3*D*). In contrast, we did not find influence of cell cycle in this context (data not shown).

The phenotype of group AP cells was detected in SHEP cells overexpressing FLIP. Accordingly, cell lines with unknown mechanism of resistance against TRAIL-induced apoptosis were examined for expression of FLIP by quantitative real-time PCR and western blot. Group AP cell lines showed higher expression of FLIP both on mRNA and protein level than all other cell lines tested (Fig. 3*E* and *F*).

Frequency of tumor necrosis factor–related apoptosis-inducing ligand–mediated proliferation in tumor cells. Of 18 nonderived tumor cell lines tested, 10 lines did not show TRAIL-induced apoptosis within 24 hours. Of these 10 cell lines, four lines belonged to group 0, whereas two belonged to group AP and four to group P (Table 1).

Primary samples of 53 children with acute leukemia were analyzed for the effect of TRAIL. Of these, 38 samples that did not show colony formation *in vitro* were analyzed for cell survival. Whereas TRAIL without his-tag (Peprotech) induced relevant apoptosis (group A) in 11 of 38 (29%) samples in accordance with the literature, TRAIL induced relevant survival (>10% specific survival) in 4 of 38 (11%) samples (group P; Fig. 4*A*). In 15 samples, tumor cells formed colonies in cell culture, which were counted under light microscopy (Fig. 4*B*). TRAIL induced relevant increase in colony formation (>120%) in 12 of 15 (80%) samples (Fig. 4*C*) which was accompanied by apoptosis induction in 3 of 15 (20%, group AP).

Taken together, in the tumor cells tested here, apoptosis induction by TRAIL was more likely in tumor cell lines (44%) compared with primary cells (21%), whereas in the latter TRAIL frequently had no effect (49%; Fig. 4*D*). TRAIL-mediated proliferation was found in nearly 25% of all cells tested, which corresponds to 40% and 31% of cells resistant against apoptosis induction by TRAIL for cell lines and primary cells, respectively.

Receptor-proximal apoptosis defects lead to tumor necrosis factor–related apoptosis-inducing ligand–mediated proliferation. For potential clinical use of TRAIL to treat patients with

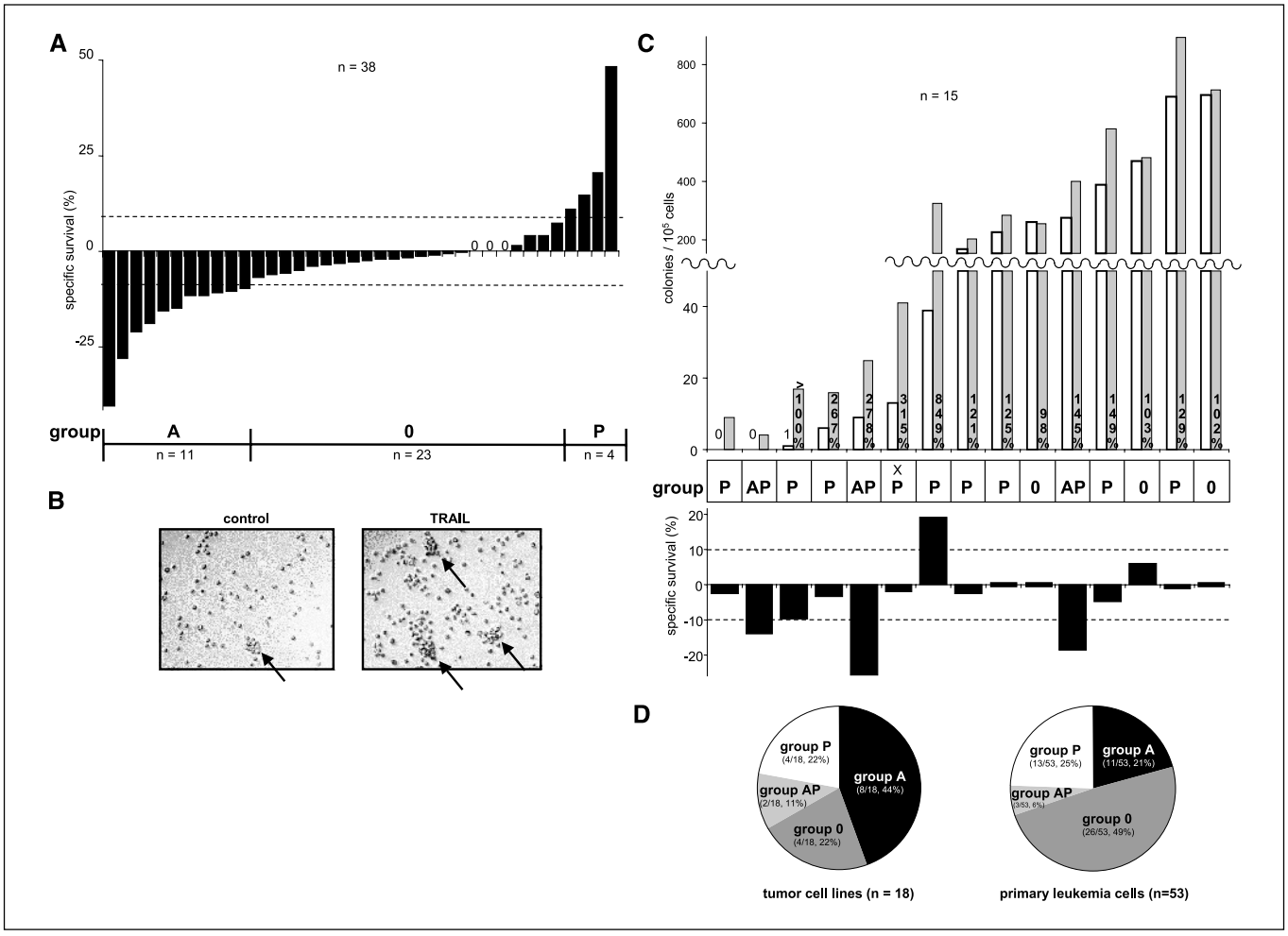

Figure 4. Effect of TRAIL on primary leukemia cells. Primary leukemia cells of 53 children with acute leukemia were isolated from bone marrow and seeded at 10^6 cells/mL in 100 μL per well in the absence or presence of TRAIL (Peprotech; without his-tag) 100 ng/mL for 24 hours. *A,* cells of 38 children did not show colony formation under any conditions. These samples were analyzed for percentage of living cells using forwardside scatter in FACScan. Data obtained for one sample. *Negative values,* TRAIL-mediated apoptosis; *positive values,* TRAIL-mediated survival. *Dashed line,* threshold for relevance for grouping (10% to −10 % specific effect of TRAIL). *B-C,* in 15 samples, colony growth was detected by light microscopy. *B,* representative microscopic view of cells of a 3.5-year-old girl with common acute lymphoblastic leukemia marked × in (*C*); colonies (*arrows*). *C,* numbers of colonies were counted in each sample in the absence (*white columns*) or presence (*grey columns*) of TRAIL; each pair of columns are results obtained in one child's primary cells. Numbers within columns indicate percentage of specific effect of TRAIL (white column was set to 100% in each patient), which was considered relevant if >120%. In parallel, cell survival was measured and depicted as in (*A*). *D,* summary of the effect of TRAIL on cell lines (as in Table 1) and primary leukemia cells (as in *A-C*).

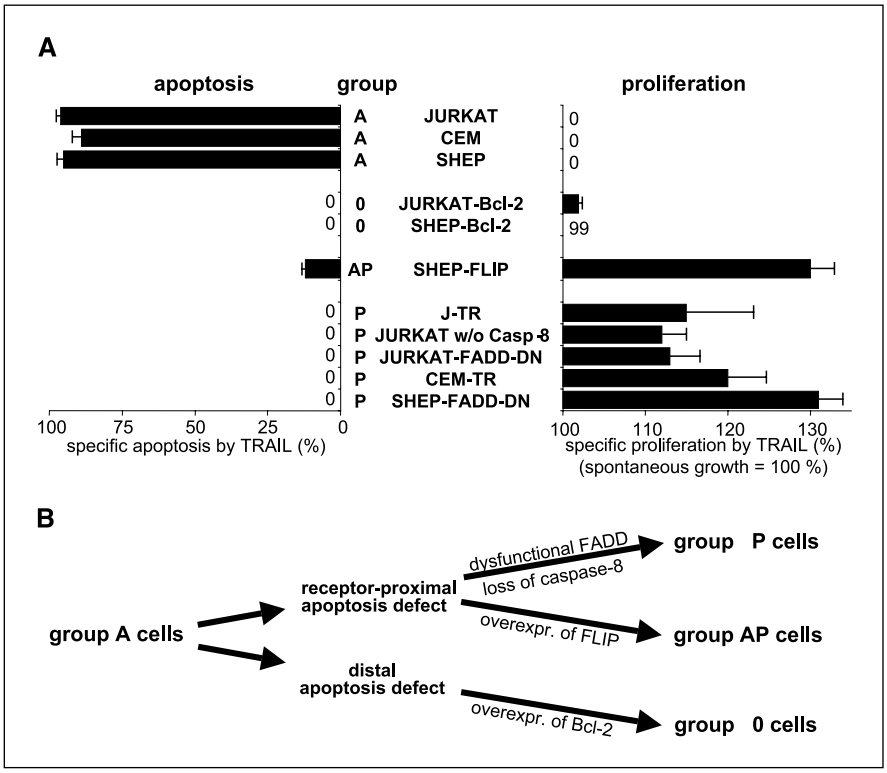

Figure 5. Mechanism responsible for TRAIL-mediated proliferation. Group A parental cell lines SHEP, JURKAT, and CEM were stably transfected with either Bcl-2, FLIP, or dysfunctional FADD (FADD-DN) or lost caspase-8 due to chemical mutagenesis (w/o Casp8) or prolonged incubation with TRAIL (J-TR, CEM-TR). Both parental and derivative cell lines were incubated with 40 ng/mL TRAIL for 24 hours. Apoptosis was measured by forwardside scatter analysis for JURKAT and CEM cells and by DNA fragmentation for SHEP cells. Proliferation was measured by Concetest in at least five independent experiments and was evaluated in the presence of zVAD-fmk in both Bcl-2-overexpressing cell lines. Proliferation is depicted as specific effect of TRAIL over control according to the numbers within the columns in Figs. 1-4. *overexpr.,* overexpressing. w/o, without. *A,* raw data; *B,* summary.

cancer, it would be helpful to predict which tumor cells will proliferate upon treatment with TRAIL and which will not. To answer this question, we tested group A tumor cell lines with acquired apoptosis defects due to stable transfection or chemical mutagenesis (Supplementary Fig. S2; ref. 10) in which apoptosis induction could be inhibited by addition of the broad spectrum caspase inhibitor zVAD-fmk (data not shown). All parental and derivative cell lines showed activation of NF-κB upon stimulation with TRAIL, including SHEP-FLIP cells (data not shown). Both cell lines overexpressing Bcl-2 did not show TRAIL-mediated proliferation even in the presence of zVAD-fmk and in the absence of apoptosis and thus belonged to group 0 (Fig. 5*A*). Loss of functional FADD by overexpression of FADD-DN as well as loss of caspase-8 or overexpression of FLIP all lead to alteration of the DISC that is formed at the TRAIL receptors. In accordance to high expression of FLIP found in group AP cells, SHEP cells overexpressing FLIP long belonged to group AP and behaved like group P cells in the presence of zVAD-fmk (Fig. 5*A*), which is dependent on expression of recombinant FLIP (Supplementary Fig. S3). Apart from SHEP-FLIP cells, all other tumor cell lines with acquired receptor-proximal apoptosis defects belonged to group P (Fig. 5*A*) as well as two nonderived cell lines (KELLY and Sy5y) lacking caspase-8. Thus, alteration in the TRAIL apoptosis signaling pathway proximal to the TRAIL receptors is associated with proliferation of tumor cells upon stimulation with TRAIL (Fig. 5*B*).

Discussion

The death ligand TRAIL as well as agonistic anti-DR4 or anti-DR5 antibodies are intensively studied as potential future cytotoxic drugs to treat malignant tumors as they induce apoptosis in a variety of different tumor cells but not in normal cells (1). We have recently described a new characteristic of TRAIL: TRAIL augments tumor cell survival and even mediates tumor cells growth of cells completely resistant against TRAIL-induced apoptosis. This function of TRAIL is independent of its preparation as it is found using either his-tagged and non-his-tagged as well as monomeric or multimerized TRAIL (data not shown; ref. 10) and is inhibited by soluble TRAIL receptor. In addition, both anti-DR4 and anti-DR5 antibodies induce proliferation of tumor cells, although both were poor agonists of apoptosis (data not shown) and were even shown to inhibit TRAIL-induced apoptosis. Here we show that tumor cells with mechanism of apoptosis resistance close to the TRAIL receptors show TRAIL-mediated tumor cell proliferation.

We classified cell lines into four groups depending on the effect of TRAIL: TRAIL induced apoptosis in group A cells, whereas it had no effect on group 0 cells, induced proliferation in group P cells and simultaneous apoptosis and proliferation in group AP cells (Fig. 3*A*). Detection of TRAIL-mediated proliferation is easily hampered by technical problems like addition of cold cell culture medium, presence of antibiotics or bacteria, or difficulties to determine cell concentration with high sensitivity and reliability. We therefore tested all cell lines in at least 10 independent experiments with at least three different methods to determine cell concentration (Fig. 1; Supplementary Fig. S1). Nevertheless, cells might have been misclassified into group 0 and A, although they belong to group P and AP, respectively, especially in primary leukemia cells where just one experiment could be done due to limited cell survival and growth in cell culture. Thus, percentage of cells belonging to group P might be underestimated due to technical limitations. Our classification was done after 24 hours of treatment with an intermediate concentration of TRAIL, whereas after, for example, 72 hours and at higher concentrations of TRAIL, all AP cells and some group 0 cells would be classified into group A.

TRAIL mediated long-term gain of tumor cells only in group P cells that represent 22% and 25% of cell lines and primary cells

tested, respectively (Fig. 4*D*). Most cytotoxic drugs prevented TRAIL-mediated proliferation of tumor cells by inducing apoptosis. In contrast, when tumor cells showed complete apoptosis resistance against both TRAIL and the cytotoxic drug, TRAIL was able to mediate proliferation in group P cells (B). Transferred into a clinical context, our data suggest that TRAIL might be unfavorable in patients with both group P tumor cells and complete apoptosis resistance against all cytotoxic drugs of a certain cycle of polychemotherapy where TRAIL might be coapplied.

Interestingly, group AP cells showed simultaneous or consecutive activation of both intracellular signaling pathways for TRAIL, the proliferation and the apoptosis pathway (Fig. 3*B*) and the equilibrium of both pathways determines the cell's fate. Accordingly, primary leukemia cells showed both apoptosis induction by TRAIL together with increase in colony growth (Fig. 4*C*). In theory, activation of both pathways might also be true for cell of groups A, but intensive apoptosis might disable measurement of proliferation. In group 0 cells, both signaling pathways might either be functional and balanced or might both be dysfunctional. Over time and in the absence of caspase inhibition, the apoptosis pathway exceeded the proliferation pathway in all group AP cells so that group AP cells behaved like group P cells at early time points and like group A cells at late time points (Fig. 3*D*). As group AP cells finally die upon treatment with TRAIL, sustained TRAIL-mediated proliferation is restricted to group P tumor cells.

All cell lines with loss of caspase-8 showed proliferation upon treatment with TRAIL. Accordingly, the broad spectrum caspase inhibitor zVAD-fmk was able to sustain TRAIL-mediated tumor cell proliferation in AP cells containing high expression of FLIP and to convert these cells into group P cells. In contrast to FLIP-transfected keratinocytes (20), SHEP-FLIP cells showed clear activation of NF-κB by TRAIL (data not shown) suggesting cell type–dependent signaling. These data suggest that the apoptosis defect caused by overexpression of FLIP might be incomplete at least at the expression level of FLIP obtained in our SHEP-FLIP cells. In contrast to group AP cells, zVAD-fmk was unable to convert group 0 cells like Bcl-2-overexpressing cells into group P cells. These data suggest that apoptosis resistance is necessary but not sufficient for TRAIL-mediated proliferation which by itself requires an intact proliferation pathway including RIP and activation of NF-κB. As dysfunction at the level of the DISC converted both SHEP and JURKAT cells into group P cells, the intracellular proliferation pathway is functional in these cell lines. In contrast, overexpression of Bcl-2 inhibited not only TRAIL-induced apoptosis but also TRAIL-mediated proliferation suggesting a role for mitochondria in both the intracellular apoptosis and the intracellular proliferation signaling pathway.

We tested eight cell lines with constitutive or acquired dysfunction at the level of the DISC: five cell lines lacking caspase-8, two cell lines with dysfunctional FADD, and one cell line overexpressing FLIP (Fig. 5). Whereas SHEP-FLIP cells showed both proliferation and apoptosis, seven of eight of these cell lines belonged to group P and showed proliferation upon stimulation with TRAIL. Thus, TRAIL-mediated proliferation is frequent among tumor cells resistant against TRAIL-induced apoptosis due to receptor-proximal apoptosis signaling dysfunction. Further studies are needed to determine whether patients bearing tumors with dysfunctional DISC should be excluded from antitumor therapy with TRAIL or antibodies directed against the TRAIL receptors DR4 and DR5.

Acknowledgments

Received 11/30/2004; revised 6/7/2005; accepted 6/17/2005.

Grant support: Wilhelm Sander-Stiftung and Bettina-Bräu Stiftung (I. Jeremias).

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We thank Daniela Föttinger for excellent technical work, Elisabeth Ronft for help with electrophoretic mobility shift assay, and Elfriede Nößner for providing renal cell carcinoma cell lines.

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