B R I E F S U M M A R Y

OF

P h D T H E S I S

ANALYSIS OF TRAIL-INDUCED APOPTOSIS IN ACUTE MYELOID LEUKEMIA CELLS

PhD Candidate: MUDr. Pavel Klener

Supervisor: Prof. MUDr. Emanuel Nečas, DrSc.

Affiliation: Institute of Pathological Physiology, 1st Faculty of Medicine, Charles University, Prague

Study Programme: Human Physiology and Pathophysiology

Chairman of the Subject Board: Prof. MUDr. Stanislav Trojan, DrSc.

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BACKGROUND AND INTRODUCTION

APOPTOSIS

Apoptosis is triggered through two major molecular pathways, designated as the **inner/intrinsic/mitochondria-mediated** and **outer/extrinsic/receptor-mediated** pathway respectively. Both pathways converge to a final common pathway resulting in the cleavage of cell regulatory and structural molecules. Both apoptotic cascades are heavily interconnected, hence the distinction in two separate pathways of apoptosis is to a certain point simplistic[1].

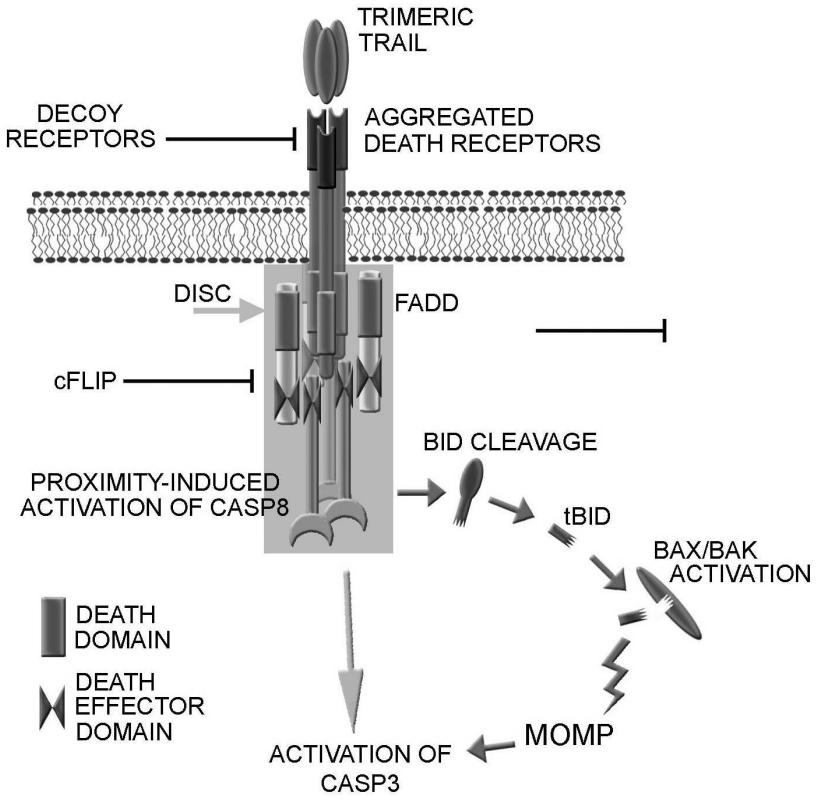
Receptor-mediated apoptotic pathway

Binding of a death ligand to the corresponding death receptor induces extrinsic, receptor-mediated pathway of programmed cell death[2]. **Death ligands** are type II membrane glycoproteins that belong to the **tumor necrosis factor (TNF) (super)family**. TNF family of cytokines is involved in the control and manipulation of the immune system. Death ligands are physiologically either membrane-bound, or proteolytically cleaved (by specific metalloproteinases) into their respective soluble forms. TNF (ligand) family comprises several protein members including TNFα, Fas-ligand (FasL, CD95 ligand), and TNF-related apoptosis inducing ligand (TRAIL, Apo2L). Receptors for TNF family ligands are type I membrane proteins that belong to the superfamily of TNF receptors (TNF-R)[3]. Based on the ability to transduce death signal the TNF-R family can be divided into **death receptors (DR)** and **decoy receptors (DcR)**. Death receptors include TNF-R1, TNF-R2, Fas (CD95), TRAIL-R1/DR4, and TRAIL-R2/DR5[4]. TNF receptor family members that lack a functional death domain (DD) and thus are unable to propagate apoptotic signal are called decoy (DcR) receptors. Membrane-bound decoy receptors include TRAIL-R3/DcR1,

and TRAIL-R4/DcR2. For decoy receptors cannot activate apoptosis they compete for specific death ligands with corresponding death receptor.

Members of the TNF ligand family (i.e. TNFα, FasL, and TRAIL) are arranged and function as homotrimers. A death ligand homotrimer binds to the trimer of corresponding TNF receptors. The receptor aggregation leads to the homophilic interaction of cytoplasmic **death domains (DD)** between the receptors and adaptor proteins **FADD** (Fas associated death domain) or **TRADD** (TNFα associated death domain). The adaptor proteins then allow recruitment of **procaspase 8** via homologous **death effector domain (DED)** motifs, which results in the assembly of a multiprotein complex called **death inducing signaling complex (DISC)**. In DISC procaspase 8 is autoactivated by so called proximity-induction mechanism. A substrate for activated caspase 8 is procaspase 3, which also represents a point of interconnection of extrinsic and intrinsic apoptotic pathways[5,6].

Receptor-mediated apoptotic pathway (legend in text)



Common apoptotic pathway

The final stages of apoptosis are characterized by typical morphological changes, such as DNA fragmentation, condensation of chromatin, proteolysis of structural proteins and cytoplasmic membrane alterations, including membrane blebbing with apoptotic bodies forming from the surface of the shrinking cell. In the course of apoptosis, phosphatidylserine is exposed from the inner to the outer leaflet of cytoplasmic membrane, which is recognized by phagocytes and leads to engulfment of apoptotic cell[7].

The executive phase of apoptosis is carried out by cysteine proteases called **caspases** (cysteine aspartic acid-specific proteases)[8]. Caspases are synthesized as inactive procaspases (zymogens). Procaspases are activated by proteolytic cleavage. Caspases create a cascade (like hemostatic one), in which the upstream caspases cleave and activate downstream caspases. Caspases can be divided into initiator/apical caspases (2, 8, 9, and 10) and effector caspases (3, 6, and 7). The initiator caspases are autoactivated during apoptosome or DISC formation during the initial phases of apoptosis by proximity-induction mechanism, and are responsible for the activation of effector caspases. Effector caspases, in coordination with initiator caspases, proteolytically cleave the cell structural proteins, mediators and regulators of apoptosis, cellular DNA repair proteins and cell cycle-related proteins, which represents terminal, irreversible phase of apoptosis.

Crosstalk

It has become evident that there is a crosstalk between the extrinsic (death receptors) and intrinsic (mitochondrial) apoptotic pathways. The best characterized connection from the extrinsic to the intrinsic pathway is mediated by the Bcl-2 family member **Bid** (BH3 interacting domain death agonist). Bid is first cleaved by active caspase 8 to yield truncated Bid (**tBid**). tBid then translocates to the mitochondrion where it binds and activates proapoptotic BH3-123 Bcl-2 members Bax and Bak. Hence, tBid crosstalk forms a

proapoptotic mitochondrial amplification loop of the receptor-mediated apoptotic pathway[9].

According to the need for tBid-mediated amplification of apoptotic signal mammalian cells can be distinguished into type I and type II cells. While in **type I cells** direct activation of caspase 3 by caspase 8 is sufficient, in **type II cells** the tBid-mediated mitochondrion apoptotic signal amplification loop is indispensable for the induction of apoptosis[10].

Regulators of apoptosis

Several important molecules antagonize with the extrinsic apoptotic pathway. **Decoy receptors** compete with death receptors for death ligands, but lack functional death domains in their intracytoplasmic portion, which precludes transduction of the apoptotic signal[11]. **cFLIP** (FLICE-inhibitory protein, FLICE= FADD-like interleukin-1-beta converting enzyme), a structural homolog of caspase 8 with enzymatically inactive pseudocaspase domain, acts as a dominant-negative competitive inhibitor of caspase-8[12].

The intrinsic and common apoptotic pathways have a wide range of modulators as well. The **Bcl-2 (B-cell lymphoma 2) family** represents probably the most important and best characterized group of mitochondrial apoptosis regulators[13].

Inhibitor of apoptosis proteins (IAP) comprise a family of at least eight proteins (cIAP1, cIAP2, NAIP, XIAP, survivin, livin etc.) that function as caspase inhibitors thereby blocking the effector phases of apoptosis[14]. The inhibitor of IAPs, *Smac/DIABLO*, is released together with cytochrom c from the mitochondrion during intrinsic apoptosis induction, which leads to enhancement of the death signal[15].

TRAIL

Death ligands

Cytokines of the tumor necrosis factor (TNF) superfamily all belong to type II membrane glycoproteins with limited homology to TNFα (overall homologies: 20%) in the extracellular region. TNFα superfamily proteins regulate development and function of the immune system. Induction of cell death is another functional feature of some members of this cytokine family, called **death ligands**. The triad of the best-explored TNF family death ligands comprises **TNFα** (tumor necrosis factor-alpha), **FasL** (Fas ligand), and **TRAIL/Apo2L** (tumor necrosis factor (TNF)-related apoptosis inducing ligand, Apo2 ligand).

TNF-related apoptosis-inducing ligand (TRAIL)

The TRAIL gene is located on chromosome 3 at position 3q26. The TRAIL monomer consists of 281 amino acids, with corresponding molecular weight of 32kD[16]. Similarly to FasL, TRAIL appears to belong among essential natural killer (NK) cell and cytotoxic T-lymphocyte weaponry against transformed, virus-infected or otherwise defected cells. Membrane-bound TRAIL (mTRAIL) can be cleaved by specific metalloproteinases to yield trimeric soluble form (sTRAIL)[17].

Both membrane-bound and soluble TRAIL induces extensive apoptosis in a wide range of tumor cell lines and primary cells, including hematologic malignancies[18]. Direct anti-tumor activities of TRAIL probably represent main physiologic role of TRAIL in the context of more complex **tumor immune-surveillance**[19]. Unlike FasL, TRAIL and its receptors are widely expressed in a variety of normal tissues, which are resistant to TRAIL-triggered programmed death[20,21]. As a consequence, strong negative regulation of TRAIL-induced apoptosis can be anticipated in most normal cells[22,23]. The TRAIL-induced tumor

cell death is usually a result of the caspase-dependent apoptotic process. Caspase 8 seems indispensable for TRAIL-induced apoptosis in most cell types, with only minor role, if any, so far reported for caspase 10. Apart from triggering apoptosis, TRAIL has also been shown to be able to induce necrotic death pathway.

Consequently, TRAIL swiftly emerged as a potential, highly specific and safe anti-cancer drug. TRAIL does not induces apoptosis of normal tissues. Despite considerable data about the safety of TRAIL it remains, however, anxiety over potential hepatotoxicity of TRAIL under specific conditions. In sharp contrast, TRAIL is a most potent apoptosis inducer in a wide range of transformed cell lines and primary tumor cells, and is capable of *in vivo* inducing tumor regression of cancer xenotransplants in immunodeficient mice, including various myelo- and lymphoproliferative diseases[24].

Receptors for TRAIL

TRAIL can bind to five receptors, which represents the most complex receptor system for one ligand in a human body[25]. Receptors for TRAIL include **death (DR) receptors** and **decoy (DcR) receptors**[26]. The TRAIL-receptor genes are all located on chromosome 8, p21-22. Both membrane-bound and soluble forms of TRAIL function as homotrimers, which results in activation of a triplet of receptors. It has been shown that the level of TRAIL death receptor expression is an independent prognostic factor in patients with several types of malignancies.

TRAIL-R1/DR4 and **TRAIL-R2/DR5** can transduce death signals upon binding of TRAIL via receptor-adaptor (DR-FADD) homologous **death domain (DD)** interaction at the intracytoplasmic receptor portion[27]. Initiator procaspase 8 is then recruited to FADD through homophilic **death effector domain (DED)** interaction, as it was described for TNFα[28]. Receptor-adaptor-procaspase 8 proteins assemble multicomponent **death inducing signaling complex (DISC)**. In the DISC, aggregated molecules of procaspase 8 undergo

proximity-induced autoactivation. The substrate for active DISC is procaspase 3[29]. In type II cells, direct cleavage of procaspase 3 by DISC is sufficient for apoptotic signal propagation. In type I cells, caspase 8-mediated cleavage of proapoptotic Bid protein represents the indispensable mitochondrial amplification loop (see above).

Besides triggering extrinsic apoptosis by activating caspase 8, ligation of TRAIL to its receptors, namely TRAIL-R1/DR4, TRAIL-R2/DR5 and TRAIL-R4/DcR2, but not TRAIL-R3/DcR3, can activate other important signaling pathways, including the generally prosurvival **NFκB**[30].

TRAIL-R3/DcR1 lacks any intracellular part, and is tethered to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor[31]. **TRAIL-R4/DcR2** has a truncated, non-functional death domain[32]. Consequently, decoy receptors cannot assemble DISC and do not convey the apoptotic signal upon binding of TRAIL. **Osteoprotegerin (OPG)**, the last of the receptors for TRAIL, is a soluble dimer that can bind TRAIL at lower affinity[33]. Decoy receptors (including OPG) thus act as competitive inhibitors of death receptors for TRAIL ligand binding.

Cytotoxic synergy of TRAIL and anti-cancer agents

Most chemotherapeutic agents used in the treatment of leukemia/lymphoma induce the mitochondrial proapoptotic pathway[34]. Resistant phenotype then often is associated with deregulation of mitochondria homeostasis, for example overexpression of Bcl-2 antiapoptotic protein. TRAIL-induced apoptosis, however, depends on caspase 8 activation rather than on the disruption of mitochondrial integrity or p53 mutation status[35]. Consequently, TRAIL retains the capacity to kill many malignant cytostatic-resistant cells. Furthermore, a great number of studies have brought evidence for additive or even synergistic cytotoxic effect of TRAIL and a variety of established or experimental anti-cancer agents[36,37]. In addition to that, combined treatment of TRAIL-resistant or

chemotherapy-resistant tumor cells with TRAIL and chemotherapy often resulted in elimination of the resistant cells[38].

Thus, combining anti-cancer agents that act on different apoptotic pathways seems to be most promising anti-tumor therapeutic strategy[39].

TRAIL: a novel therapeutic agent?

Several soluble recombinant forms of human TRAIL have been prepared up to now. A polyhistidine-tagged soluble form (**His-TRAIL**, amino acids 114-281), leucine zipper-linked TRAIL (**LZ-TRAIL**, amino acids 95-281), or **rhTRAIL** devoid of any additional foreign sequence (amino acids 114-281), respectively, have all shown substantial biologic activity *in vitro* and *in vivo*[18,24]. Phase I safety and pharmacokinetic study of recombinant TRAIL (Genentech, Amgen) in patients with advanced cancers has clearly shown TRAIL is safe and well tolerated up to 15 mg/kg, given in the form of intravenous 60 minute infusion. Contribution of TRAIL to the standard treatment of advanced solid tumors and hematological malignancies will be evaluated in randomized trials in the upcoming years.

Mechanisms of tumor resistance to TRAIL

Unfortunately, not all malignant cells are sensitive to TRAIL-induced apoptosis. Numerous mechanisms of resistance to TRAIL have been reported up to the present[40]. **Somatic mutations of TRAIL-R1/DR4 and TRAIL-R2/DR5 genes** may play a role in the pathogenesis of some non-Hodgkin´s lymphomas (NHLs)[41]. It is speculated that genes encoding TRAIL-R1/DR4 and TRAIL-R2/DR5 act as dosage-dependent tumor suppressor genes, whose monoallelic deletion can impair TRAIL-induced apoptosis in B-cell lymphomas[42]. **Overexpression of membrane decoy receptors (TRAIL-R3/DcR1 and/or TRAIL-R4/DcR2)** has been correlated with resistance to TRAIL in acute myeloid leukemia and pancreatic cancer cells[11]. The role of **soluble osteoprotegerin** for tumor cell

survival remains largely elusive, but recent studies insinuate OPG might function as a paracrine survival factor for the tumor cells[43] Disruption of DISC components, namely **caspase 8** and **FADD**, have been reported responsible for constitutive or acquired TRAIL resistance of tumor cells[44]. **Overexpression of cFLIP** represents one of the best-characterized mechanisms associated with TRAIL and FasL resistant phenotypes[45]. Increased expression of antiapoptotic family proteins **IAPs** (in particular **XIAP**) conferred TRAIL resistance to a wide range of malignant cells[46]. Deregulation of **Bcl-2 family members**, especially enhanced transcription of Bcl-2, Bcl-xL, or Mcl-1, contributed to TRAIL-resistant phenotype in several tumor cell lines[47]. TRAIL-resistance of tumor cells was frequently associated with deregulation of important cell signaling pathways, in particular with **aberrant activation of prosurvival signaling pathways NFκB**[48].

A number of diverse molecules responsible for tumor cell TRAIL resistance have been reported up to the present, emphasizing that extreme complexity applies not only to TRAIL-induced apoptotic pathway, but also to the mechanisms that lead to its obliteration.

EXPERIMENTAL STUDY

AIMS OF THE STUDY

I. To identify molecular mechanisms responsible for acquired resistance of TRAIL-sensitive HL60 leukemia cells to TRAIL-induced apoptosis following prolonged *in vitro* treatment with TRAIL.

IIa. To determine whether acquired TRAIL resistance of HL60 leukemia cells is associated with resistance to other cytotoxic agents, namely to other death ligands (TNFα*) and to cytostatics currently used for the treatment of acute leukemia (cytarabine, idarubicin).

IIb. To specify whether acquired resistance of HL60 leukemia cells to cytostatics (cytarabine, idarubicin) impacts sensitivity/resistance to TRAIL.

III. To measure and compare cytotoxic effects of a combined *in vitro* treatment using TRAIL and currently used anti-leukemia cytostatic agents, cytarabine and idarubicin, on proliferation and survival of HL60 leukemia cells.

* Fas ligand was not tested because HL60 leukemia cell line (despite expressing Fas) is FasL-resistant[49]

MATERIALS AND METHODS

Cell culture conditions, antibody staining, TRAIL-induced apoptosis measurement

Recombinant his-TRAIL was expressed in E. coli BL-21 and purified by the sequential chromatography on TALON (Clontech, Palo Alto, CA, USA) and SPSepharose (Pharmacia, Peapack, NJ, USA). The expression of TRAIL receptors was measured by flow cytometry (FACS Calibur; Becton Dickinson, CA, USA) using monoclonal antibodies (R&D systems, MN, USA). TRAIL receptors were blocked with polyclonal antibodies (Apronex Biotechnologies, Czech Republic; R&D systems, MN, USA). Apoptosis was analyzed by flow cytometry (FACS Calibur) using Annexin-V-FITC staining (Apronex Biotechnologies, Czech Republic). The blockage of apical caspases 8 and 10 was achieved by specific caspase inhibitors (R&D systems, MN, USA).

Establishment of TRAIL-resistant and cytostatic-resistant HL60 cells

TRAIL-resistant HL60 cell *lines* were derived by selective pressure of TRAIL (200ng/ml, Apronex Biotechnologies, Czech Republic). Cytostatic-resistant HL60 cell lines were selected by cultivating cells with increasing doses of idarubicin (Zavedos, Pharmacia, Italy, 2nM to 30nM) and cytarabine (Cytosar, Pharmacia&Upjohn, Belgium, 0.1μM to 2mM). Proportion of TRAIL-resistant cells in the WT HL60 population was detected by limiting dilution assay.

RNA extraction, cDNA reverse transcription and real-time RT-PCR analysis

Real-time PCR analysis was performed using IQ^{TM}5 multicolor real-time PCR detection system (Bio-Rad, USA). The results were normalized to actin mRNA expression.

Measurement of Caspase 8 and 10 Activation in response to TRAIL and TNFα

Caspase activation was measured by Caspase8or10/FLICE fluorometric assays (R&D systems, MN, USA) according to the manufacturer's protocol. The fluorescence intensity was measured by Plate CHAMELEON fluorescence reader (Hidex, Finland).

Detection of NFκB translocation

NFκB RelA/p65 translocation was measured using TransAm™ ELISA-based NFκB RelA/p65 activation assay (Active Motif, Belgium) according to the manufacturer's instructions.

Xenotransplantations of HL60 cells into immunodeficient NOD/LtSz-Rag1^{null} *mice*

NOD/LtSz-Rag1^{null} female mice aged 8 to 12 weeks were 4Gy γ-irradiated on day 0. The cells were injected into tail veins of the irradiated animals on day 1. Each group of mice comprised six animals. The mice were daily observed and were sacrificed when developed hind-leg paralysis, or appeared severely sick. FITC-conjugated anti-human CD45 antibody was used to measure the extent of potential leukemic infiltration of murine tissues (bone marrow, liver, spleen, brain, tumors).

Measurement of synergistic cytotoxic effects of TRAIL, cytarabine and idarubicin on TRAIL-sensitive WT HL60 cells

WT HL60 (1000 cells / well) were distributed onto 96-well plate, each well corresponding to one "leukemia unit". On day 1, several concentrations and/or combinations of TRAIL, idarubicin (IdaR), and cytarabine (AraC), each representing a different treatment strategy, were added to the "leukemia units" in attempt to eradicate HL60 cells. On day 8, twelve wells of each treatment strategy plates were analyzed by MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) proliferation assay according to the manufacturer's protocol. On day 30, the remaining leukemia units were examined under light microscope, and the leukemia units that contained proliferating cells were marked as 1 (=progression/relapse of leukemia), while the units that lacked living cells were marked as 0 (=complete eradication of leukemia).

RESULTS

Characterization of TRAIL-induced apoptosis of HL60 cells

Both TRAIL and TNFα-induced apoptosis of HL60 cells is predominantly a caspase dependent process. Treatment with specific caspase inhibitors significantly suppressed TRAIL or TNFα-induced apoptosis in HL60 cells. However, in HL60 cells, unlike other cell lines tested (data not shown), caspase inhibition did not result in complete abrogation of TRAIL or TNFα-induced apoptosis (Fig.2). The data thus suggest TRAIL and TNFα-induced death signaling might, at least partially, be mediated by caspase-independent apoptotic pathways.

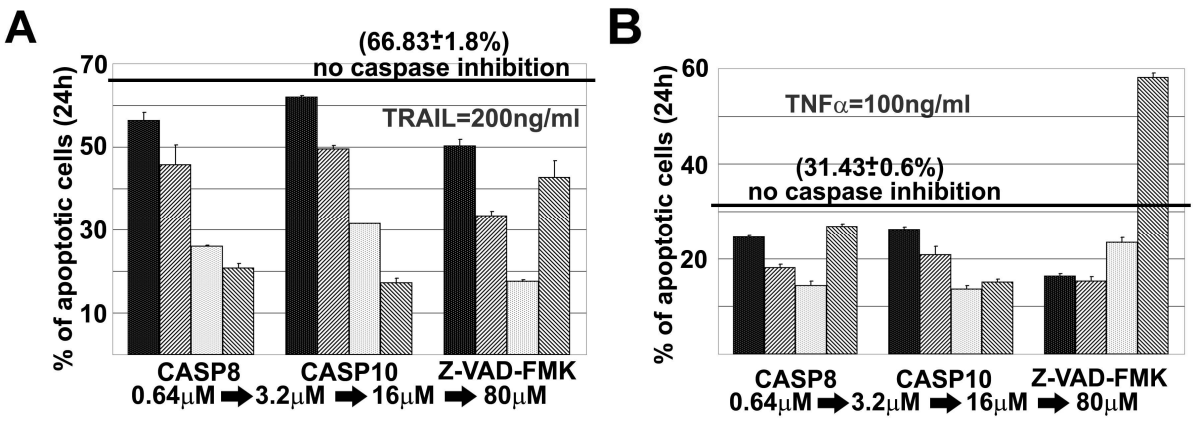


Figure 1: Caspase 8 inhibitor Z-IETD, caspase 10 inhibitor Z-AEVD and pan-caspase inhibitor Z-VAD-FMK suppressed, but did not completely abrogate, TRAIL-induced (A) or TNFα-induced (B) apoptosis of HL60 cells. Z-VAD-FMK at high concentrations increased numbers of TRAIL or TNFα-induced HL60 apoptotic cells by compound-specific mechanism. Caspase inhibitors (0.64, 3.2, 16 and 80uM) were added to HL60 cells 1 hour before addition of TRAIL (200ng/mL) or TNFα (100ng/mL). Level of apoptosis was analyzed after 24 hours by flow cytometry using FITC-conjugated Annexin-V. Columns represent means, and error bars standard deviations of values of three independent experiments minus background (i.e. apoptosis induced by caspase inhibitor(s) only). "no caspase inhibition" line represents % of apoptotic cells induced by TRAIL (200ng/ml) or TNFα (100ng/ml) minus background (i.e. spontaneous apoptosis).

Antibody-mediated blockage of WT HL60 death receptor TRAIL-R1/DR4 more potently suppressed TRAIL-induced apoptosis than blockage of TRAIL-R2/DR5 (Fig.3).

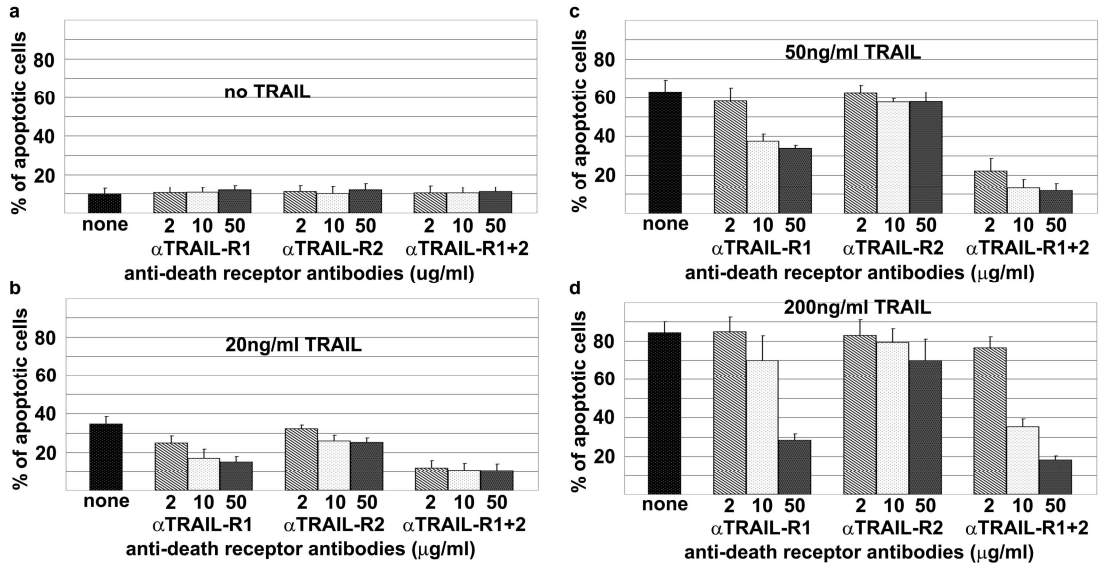


Figure 2: TRAIL-R1/DR4 transduces TRAIL apoptotic signal more potently than TRAIL-R2/DR5 in WT HL60 cells. The cells were pre-incubated with blocking antibodies against death receptor TRAIL-R1/DR4 (αTRAIL-R1), TRAIL-R2 (αTRAIL-R2), or both receptors (αTRAIL-R1+2) before exposure to TRAIL (20, 50, 200ng/mL). Level of apoptosis was analyzed by flow cytometry 24h after addition of TRAIL using FITC-conjugated Annexin-V assay. Columns represent means, and error bars standard deviations of values of three independent experiments.

HL60 TRAIL-resistant cells are resistant to TNFα, but remain sensitive to idarubicin and cytarabine

TRAIL-resistant HL60 cell lines (n=20) were derived by selective pressure of TRAIL. All TRAIL-resistant HL60 cell lines were resistant to TRAIL-induced apoptosis compared to WT HL60 cells.

TNFα, anti-Fas agonistic antibody, cytarabine, and idarubicin were used to determine sensitivity/resistance of the TRAIL-resistant cells to other cytotoxic agents compared to WT HL60 cells. TNFα-induced apoptosis was suppressed in TRAIL-resistant HL60 cell lines compared to WT HL60 cells. HL60 cells, despite expressing cell surface Fas receptor, are known to be resistant to FasLigand-induced apoptosis. The levels of apoptosis induced

by cytotoxic agents, cytarabine and idarubicin, were not significantly different between TRAIL-resistant and WT HL60 cells.

Acquired TRAIL resistance of HL60 cells is associated with resistance to TNFα, but not with resistance to cytotoxic agents, idarubicin or cytarabine. Receptor-mediated apoptotic pathway, unlike mitochondrial apoptotic pathway, thus seems to be significantly disrupted in TRAIL-resistance HL60 cells.

HL60 cells resistant to cytarabine or idarubicin remain sensitive to TRAIL and TNFα

By using selective pressure of cytarabine (AraC) or idarubicin (IdaR) we derived cytarabine or idarubicin-resistant HL60 cell lines. Both AraC and IdaR-resistant HL60 cells remained sensitive to TRAIL and TNFα-induced apoptosis. Acquired resistance to cytostatics was not associated with significant alterations of receptor-mediated apoptosis in HL60 cells.

Two distinct phenotypes of TRAIL-resistant HL60 cells

TRAIL receptors, TRAIL, and CD14 were analyzed in TRAIL-resistant and WT HL60 cells by flow cytometry and real-time RT-PCR. Two distinct TRAIL-resistant phenotypes, designated as phenotypes P1 and P2, were identified (Fig.3, Fig.4). P1 was detected in 17 of 20 (85%), and P2 in 3 of 20 (15%) TRAIL-resistant HL60 cell lines.

P1 compared to WT HL60 cells showed prominent downregulation of both cell surface TRAIL death receptors, and of decoy receptor TRAIL-R3/DcR1 (Fig.7). The cells also demonstrated moderate downregulation of cell surface decoy receptor TRAIL-R4/DcR2, while the expression of CD14 did not significantly change compared to WT HL60 cells (Fig.3, Fig.4).

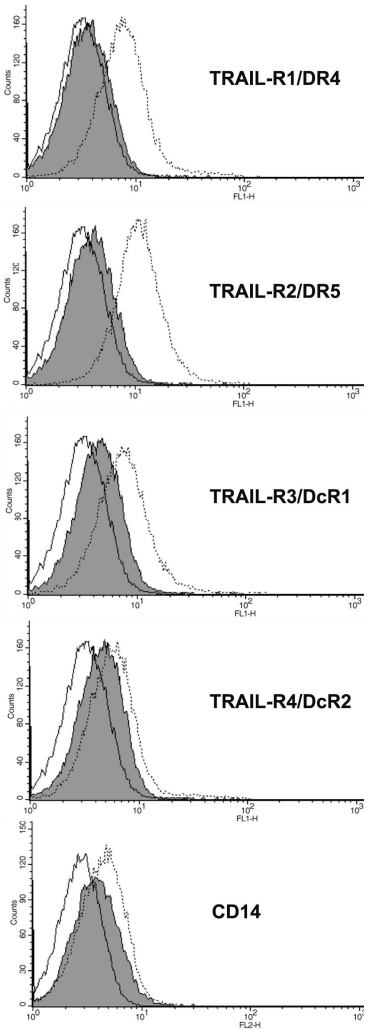


Figure 3: Cell surface expression of TRAIL receptors and CD14 in TRAIL-resistant P1 HL60 cells. TRAIL death receptors and decoy receptor 1 (TRAIL-R1, 2, 3) are downregulated, while expression of decoy receptor 2 (TRAIL-R4) does not change in P1 compared to WT HL60 cells. Cells were stained with goat-anti-human-TRAIL-receptor polyclonal antibody, and subsequently with FITC-conjugated anti-goat secondary antibody before analyzed by flow cytometry. Full histograms represent TRAIL receptor and CD14 stains of the P1 cells. Interrupted open histograms represent TRAIL receptor and CD14 stains stains of the WT HL60 cells. Continuous open histograms represent control stains of the P1 cells with secondary antibody only. Data presented are representative example of P1 HL60 cell line phenotype.

P2 cells showed downregulation of both TRAIL death receptors, while the expression of TRAIL-R3/DcR1 and TRAIL-R4/DcR2 did not significantly change. Surface myeloid differentiation CD14 antigen was induced in P2 compared to both WT and P1 HL60 cells (compare Fig.3 and 4). Neither TRAIL nor OPG were detectable by flow cytometry on the cell surface of HL60 cells (data not shown).

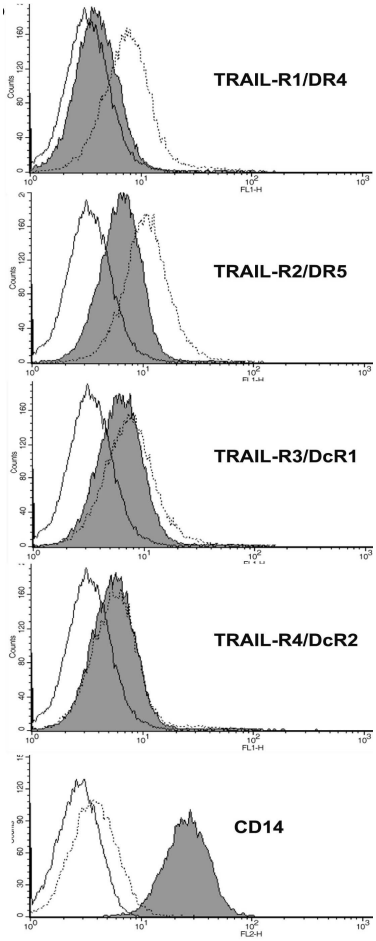


Figure 4: Cell surface expression of TRAIL receptors and CD14 in TRAIL-resistant P2 HL60 cells. TRAIL death receptors (TRAIL-R1, 2) are downregulated, while expressions of decoy receptors (TRAIL-R3, 4) do not change in a significant way in P2 compared to WT HL60 cells. Cells were stained with goat-anti-human-TRAIL-receptor polyclonal antibody, and subsequently with FITC-conjugated anti-goat secondary antibody before analyzed by flow cytometry. Full histograms represent TRAIL receptor and CD14 stains of the P2 cells. Interrupted open histograms represent TRAIL receptor and CD14 stains of the WT HL60 cells. Continuous open histograms represent control stains of the P2 cells with secondary antibody only. Data presented are representative example of P2 HL60 cell line phenotype.

Receptors for TNFα are downregulated in all TRAIL-resistant HL60 cell lines

All TRAIL-resistant HL60 cell lines were resistant to TNFα-induced apoptosis. The cell surface expression of receptors for TNFα was significantly downregulated in these cell populations (Fig.5).

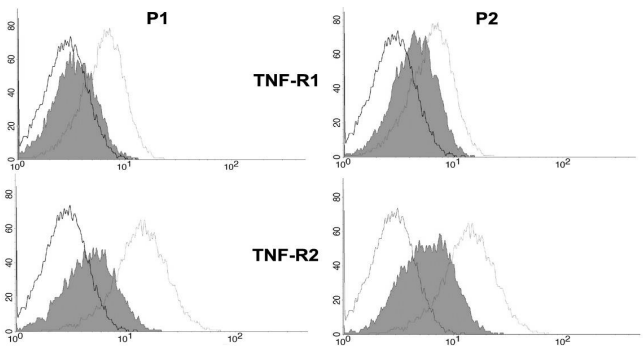


Figure 5: Cell surface expression of receptors for TNFα is significantly downregulated in TRAIL resistant HL60 cells of both phenotypes (P1 and P2= full histograms) compared to WT HL60 cells (=continuous open histograms). Cells were stained with goat-anti-human TNFα-receptor polyclonal antibody, and subsequently with FITC-conjugated anti-goat secondary antibody before analyzed by

flow cytometry. Full histograms represent TRAIL receptor and CD14 stains of the P1 and P2 HL60 cell lines. Continuous open histograms represent TRAIL receptor and CD14 stains of the WT HL60 cells. Interrupted open histograms represent control stains of the P1 and P2 HL60 cell lines with secondary antibody only. Data presented are representative examples of P1 and P2 HL60 cell line phenotypes.

TRAIL and TNFα–induced caspase 8 and 10 activation is inhibited in TRAIL resistant HL60 cells compared to WT HL60 cells.

The activation of caspase 8 and 10 after the exposure of TRAIL was significantly decreased in TRAIL resistant HL60 lines compared to WT HL60 cells. While in P2 cells we did not detect any caspase 8 activation during the first 4 hour after the exposure to TRAIL, in P1 cells the caspase 8 activation was significantly lower compared to HL60 WT cells. Interestingly, in WT HL60 cells the increase of caspase 10 activity after both TRAIL and TNFα was higher compared to the increase of caspase 8.

Specific expression patterns of proapoptotic and antiapoptotic molecules are associated with P1 and P2 TRAIL resistant HL60 phenotypes

To find possible molecular mechanisms that might contribute to acquired TRAIL-resistance in HL60 cells, we analyzed gene expressions of selected molecules known to potently intefere with the apoptotic process. Gene expression changes of P1 compared to WT HL60 cells, especially significant overexpression of IAP and BCL2 family members, might at least partially contribute to the acquired TRAIL-resistance. Gene expression changes of the P2 cells were mostly proapoptotic. However, the marked downregulation of FADD might synergize with downregulation of TRAIL (and TNFα) death receptors in completely shutting down the extinsic apoptotic pathway (Fig.6).

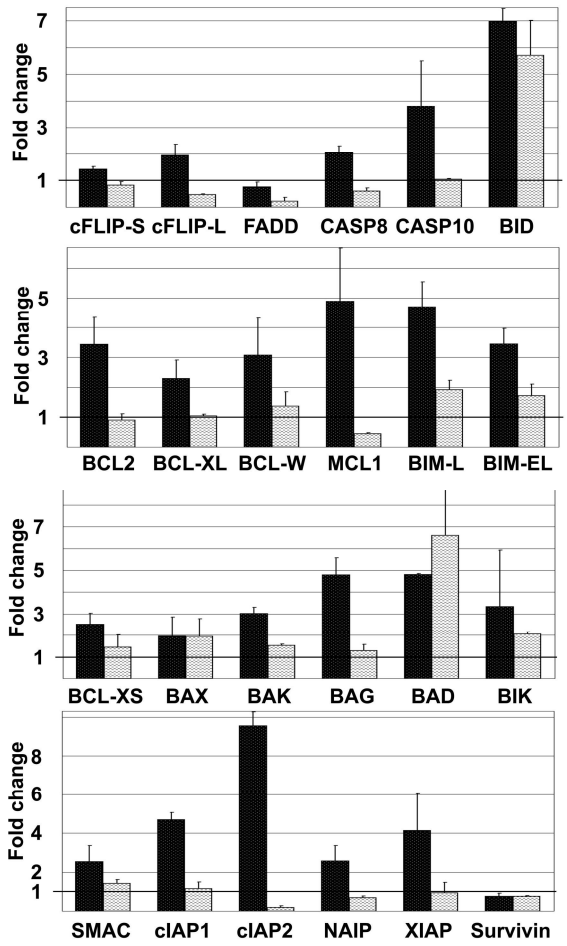


Figure 6: The expression of several essential apoptosis regulators is altered in P1 and P2 compared to WT HL60 cells as detected by real-time RT-PCR analysis. Figure 6 shows relative amounts of mRNA in P1 (dark columns), and P2 (light columns) HL60 cells compared to relative amount of mRNA in WT HL60 cells (=1). WT HL60 cells and two cell lines from each TRAIL-resistant phenotype were analyzed. Gene expressions of selected pro- and antiapoptotic molecules were analyzed. Columns represent means, and error bars standard deviations of values of four independent experiments (two experiments for each cell lines).

Diminished TRAIL-induced NFκB nuclear translocation in both TRAIL resistant HL60 phenotypes compared to WT HL60 cells

TRAIL-induced translocation of RelA/p65 transcription factor to the nucleus of TRAIL-exposed TRAIL-resistant compared to WT HL60 cells was significantly suppressed. This might be the result of the downregulation of cell surface TRAIL receptors as well as by the defect in TRAIL receptor signaling pathways. The role of prosurvival NFκB pathway in acquired TRAIL-resistance was, however, not confirmed.

Shorter survival of immunodeficient mice xenotransplanted with P2 compared to mice xenotransplanted with WT or P1 HL60 cells

Intravenous injection of WT HL60 cells into immunodeficient NOD/LtSz-Rag1^{null} mice with subsequent leukemia development was associated with massive brain leukemia

cell infiltration. The infiltration of bone marrow, liver, and peripheral lymph nodes was frequent. WT HL60 injected mice did not develop either hind-leg paralysis or macroscopic intraperitoneal tumors. Xenotransplantation of P1 cells did not significantly differ from that of WT HL60 cells. In contrast, the injection of P2 cells was associated not only with constant brain infiltration, and frequent bone marrow, liver and peripheral lymph node infiltration, but also with multiple intraperitoneal tumors and occasional hind-leg paralysis. Survival of the mice xenotransplanted with P2 cells was significantly shorter (30.8 ± 2 days) compared to both WT and P1 cells transplanted mice (41.3 ± 2.1 and 40.5 ± 1.5 days, respectively; $p \leq 0.01$).

TRAIL potentiates cytotoxic effects of cytarabine and idarubicin in WT HL60 cells

We measured the effects of diverse therapeutic strategies comprising different combinations and/or concentrations of the TRAIL, cytarabine (AraC) and idarubicin (IdaR), on WT HL60 cell proliferation and survival.

Our results proved significant cytotoxic synergy of TRAIL with either idarubicin, cytarabine or both agents. These data might have important implication for the development of new therapeutic strategies in the therapy of acute myeloid leukemia.

DISCUSSION

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is a cytokine with tumor-specific proapoptotic activity. In the present study we designed an *in vitro* model of acquired TRAIL resistance of leukemic cells. TRAIL sensitive myeloid leukemia HL60 cells were exposed to selective pressure of TRAIL, and the established TRAIL-resistant HL60 cell lines were analyzed.

Prolonged exposure of WT HL60 cells to TRAIL resulted in the expansion of several TRAIL-resistant HL60 cell lines. Analysis of the TRAIL-resistant lines unveiled that they could be divided into two TRAIL-resistant phenotypes: P1 cells (detected in 17 of 20 HL60 TRAIL-resistant lines) and less frequent P2 cells (detected in 3 of 20 lines). P2 cells showed gain of CD14 differentiation antigen expression and loss of osteoprotegerin (OPG) expression compared to both P1 and WT HL60 cells. TRAIL has recently been reported to induce myeloid differentiation of normal human myeloid cells[50]. Maturation marker CD14 is induced during myeloid differentiation. The overexpression of CD14 on P2 cells thus might imply that this change was induced by TRAIL. The discrimination of TRAIL resistant HL60 cell lines into two phenotypes, P1 and P2, might imply specific mechanisms of TRAIL resistance development.

Recently, acquired resistance to TRAIL in human ovarian carcinoma cell line was associated with resistance to Fas-ligand (FasL), but not to chemotherapeutic agents[51]. We demonstrated that acquired TRAIL resistance of HL60 leukemia cells was associated with resistance to TNFα., but not to anti-leukemia agents, cytarabine or idarubicin. Resistance to TNFα was associated with significant downregulation of TNFα receptor cell surface expression. To analyze potential disruption of apical events of receptor-mediated apoptosis, we measured the ability of TRAIL and TNFα to induce caspase 8 and 10 activation in

TRAIL resistant and WT HL60 cells. In WT HL60 cells blockage of either caspase 8 or caspase 10 led to the significant abrogation of TRAIL or TNFα-induced apoptosis. TRAIL resistant HL60 cell lines had decreased activities of both caspase 8 and 10 after the challenge with TRAIL or TNFα, which is in concordance with decreased expression of TRAIL and TNFα receptors. Interestingly, in WT HL60 cells the increase of caspase 10 activity after both TRAIL and TNFα was higher compared to the increase of the activity of caspase 8. The data show that not only caspase 8 but also caspase 10 seems to play an essential role in death receptor-mediated apoptosis of HL60 cells.

TRAIL has recently been reported to induce growth of a subset of leukemic cells, and to enhance metastatic progression of pancreatic cancer[52,53]. In the present study, TRAIL did not stimulate growth of TRAIL-resistant HL60 cells *in vitro* (data not shown). However, xenotransplantation of P2 HL60 cells into immunodeficient NOD/LtSz-Rag1^{null} mice was associated with more aggressive biologic behavior and different growth pattern compared to both P1 and WT HL60 cells. The results suggest that acquired leukemia cell TRAIL-resistance might at least in some cases be associated with more aggressive biologic behavior and ensuing worse prognosis.

We clearly showed that single-agent TRAIL was not able to completely eradicate WT HL60 leukemia cells. Combined treatment of diverse tumor cells with TRAIL and other anti-cancer agents had additive or even synergistic cytotoxic effect in many experimental studies[36]. Data from our own experiments demonstrate significant synergistic cytotoxic effect between TRAIL, cytarabine and idarubicin on HL60 leukemia cells. This finding is of special importance, as combined application of TRAIL and currently used cytostatics might significantly improve outcome of patients with acute myeloid leukemia.

CONCLUSIONS

1. TRAIL-induced apoptotic signal is conveyed predominantly via TRAIL-R1/DR4 in WT HL60 cells.

2. Prolonged treatment of TRAIL-sensitive HL60 leukemia cells with TRAIL resulted in the establishment of TRAIL resistant HL60 cell lines. Two distinct phenotypes of acquired TRAIL resistance, designated P1 and P2, were identified among TRAIL-resistant HL60 cell lines, based on the expression of TRAIL receptors, CD14, selected apoptotic regulators, nuclear translocation of NFκB p65/RelA, and biologic *in vivo* aggressivity. **Different molecular mechanisms are involved in acquired TRAIL-resistance of HL60 cells.**

3. TRAIL resistance of HL60 cells was associated with resistance to TNFα, but not to the cytostatics, idarubicin and cytarabine. **Cytarabine-resistant and idarubicin-resistant HL60 cells remained sensitive to TRAIL and TNFα.** The extrinsic apoptotic pathway is disrupted in TRAIL resistant HL60 cells. The intrinsic apoptotic pathway, however, does not seem to be significantly affected by TRAIL resistance development.

4. Acquired resistance of HL60 cells to TRAIL was associated with cell surface downregulation of death receptors for TRAIL and TNFα. The detected downregulation of the death receptors in TRAIL-resistant HL60 cells might represent a major molecular mechanism of TRAIL and TNFα resistance.

5. TRAIL and TNFα-induced caspase 8 and caspase 10 activation was suppressed in TRAIL resistant compared to WT HL60 cells. This could be a consequence of TRAIL and TNFα receptor downregulation from the cell surface, or an alteration of other molecules of the TRAIL receptor signaling pathways

6. Changed expression of apoptotic regulators, namely the detected downregulation of **FADD**, and upregulation of **IAP** and **BCL2** family members in TRAIL resistant compared to WT HL60 cells, **might contribute to TRAIL resistance of HL60 cells**,.

7. NFκB p65/RelA translocation to the nucleus is suppressed in TRAIL resistant compared to WT HL60 cells. **NFκB prosurvival pathway is not implicated in acquired TRAIL resistance** of HL60 cells.

8. Acquired TRAIL resistance of leukemia cells might be associated with increased *in vivo* aggressivity, as shown on WT, P1 and P2 HL60 cell xenograft model.

9. TRAIL potentiates cytotoxic effect of cytarabine and idarubicin on HL60 leukemia cells. This could have important implications for the treatment of leukemia.

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APPENDIX

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