P h D T H E S I S

ANALYSIS OF TRAIL-INDUCED APOPTOSIS IN ACUTE MYELOID LEUKEMIA CELLS

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EXPERIMENTAL STUDY

DECLARATION

The thesis was worked out at the Institute of Pathological Physiology, Prague, from 2004-2007.

I hereby declare that I have worked out the thesis independently while noting all the resources employed as well as co-authors. I consent to the publication of the thesis under Act No. 111/1998, Coll., on universities, as amended by subsequent regulations.

Prague, 1.9.2007 MUDr. Pavel Klener

ABBREVIATIONS:

AIF= apoptosis inducing factor

AML= acute myelogenous leukemia

AraC= cytarabine

BCL2= B-cell lymphoma 2

BID= Bcl-2 interacting domain death agonist

BH= Bcl homology

CASP= caspase= cysteine aspartic acid-specific protease

CML= chronic myelogenous leukemia

DcR= decoy receptor

DD= death domain

DED= death effector domain

DISC= death inducing signaling complex

DR= death receptor

FACS= fluorescence-activated cell sorter

FADD= Fas-associated death domain

FasL= Fas ligand

FBS= fetal bovine serum

FITC= fluorescein isothiocyanate

FLICE= FADD-like interleukin-1β converting enzyme

FLIP= FLICE-inhibitory protein

His-TRAIL= polyhistidine-tagged TRAIL

HSP= heat-shock protein

IAP= inhibitor of apoptosis

IdaR= idarubicin

IMDM= Iscove´s modified Dulbecco´s medium

JNK= Jun N-terminal kinase

MDS= myelodysplastic syndrome

MOMP= mitochondrial outer membrane permeablization

MTT= 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide

NFκB= nuclear factor kappa B

NHL= non-Hodgkin lymphoma

NOD/LtSz-Rag1^{null} = non-obese diabetic / recombination-activating gene 1 null mice

(rh)OPG= (recombinant human) osteoprotegerin

SMAC/Diablo= second mitochondria-derived activator of caspase / Direct IAP binding protein with low pI

tBID= truncated BID

TNFα= tumor necrosis factor alpha

TRADD= TNF receptor-associated death domain

TRAF= TNF receptor associated factor

(rh)TRAIL= (recombinant human) TNF-related apoptosis-inducing ligand

WT= wild-type

XIAP= X-linked inhibitor of apoptosis

BACKGROUND AND INTRODUCTION

I. APOPTOSIS

Apoptosis and necrosis represent two major mutually different mechanisms of cell death [1]. **Necrosis** is defined as uncontrolled process of cellular break-up executed as a consequence of massive and irreversible damage. Necrosis leads to the release of inflammatory cytokines [2]. **Apoptosis** is controlled, to a certain degree reversible process of programmed cell disintegration that usually doesn't induce inflammatory reaction. Although the understanding of detailed signaling pathways that execute apoptosis is incomplete, this process is controlled by numerous proteins and protein complexes that are activated by various triggers which induce sequential activation of effector mechanisms [3].

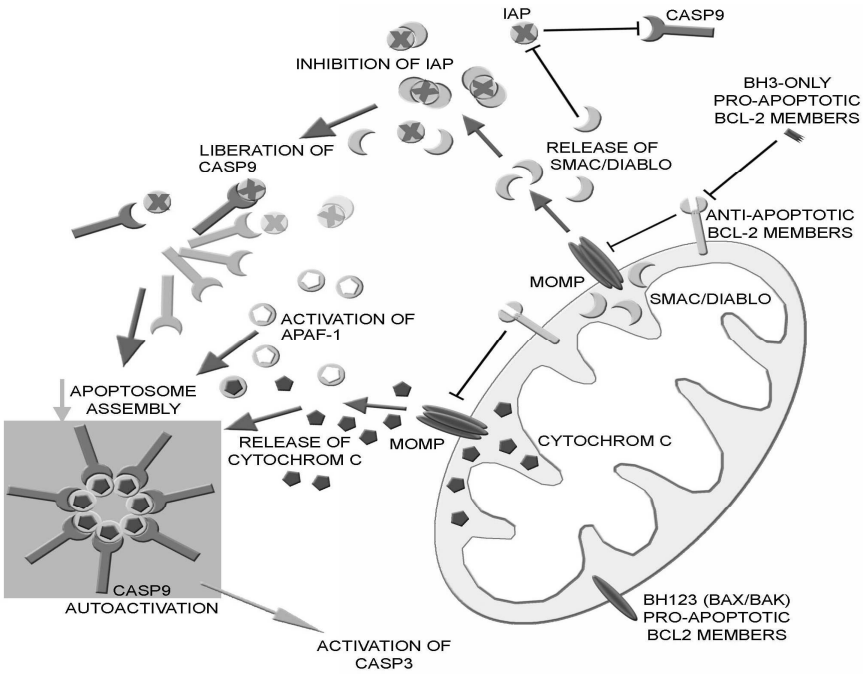
Apoptosis is triggered through two major molecular pathways, designated as the **inner/intrinsic/mitochondria-mediated** and **outer/extrinsic/receptor-mediated** pathway respectively [4-8]. Both pathways converge to a final common pathway resulting in the cleavage of cell regulatory and structural molecules. Both apoptotic cascades are heavily interconnected, hence the distinction in two separate pathways of apoptosis is to a certain point simplistic. Apoptosis is an essential part of life for multicellular organisms important for proper embryonic development and maintenance of adult tissue homeostasis. The defect in apoptosis and in apoptotic regulatory mechanisms can result in various pathological states, including malignant transformation, or tumor progression. In transformed cells loss of sensitivity to apoptosis represents one of the key molecular mechanisms of resistance to chemo/immuno/radiotherapy [9]. The understanding of apoptosis and its regulatory

mechanisms is essential for the understanding of pathogenesis of malignant disorders as well as the development of more effective therapies.

a. Mitochondrial apoptotic pathway

The intrinsic apoptotic pathways are initiated inside the cell [7]. The factors involved in the intrinsic apoptotic pathway induction are physical and chemical injuries (e.g. UV radiation, heat shock, osmotic shock, hypoxia), changed expression of cellular oncogenes and tumor suppressor genes (e.g. cMyc, cFos, p53, PTEN), disruption of cytoskeleton structures, DNA damage (e.g. mutagenic agents, cytostatics, antimetabolites), growth factors/cytokines deprivation, nucleotide/ATP deficiency and other stress factors. While the initial triggers of diverse intrinsic apoptotic pathways may differ substantially, all of them inevitably converge to the pivotal event, which is the **mitochondrial outer membrane permeabilization (MOMP)** [10]. MOMP is associated with mitochondrial inner transmembrane potential dissipation (ΔΨm). Antiapoptotic Bcl-2 family members function to block MOMP, whereas various proapoptotic Bcl-2 members promote it [10-12].

Figure i: Mitochondrial apoptotic pathway (legend in text)



Subsequently, **cytochrom c** together with other proapoptotic molecules Apaf-1 (apoptotic protease-activating factor 1), Smac/DIABLO (second mitochondria derived activator of caspase / Direct IAP binding protein with low pI), AIF (apoptosis inducing factor), and endonuclease G are released to the cytoplasm from the disrupted mitochondria [13-18]. **Apaf-1**, an adaptor protein, binds (in the presence of ATP/dATP) cytochrom c and **procaspase 9** to form a multiprotein complex, referred to as the **apoptosome** [19-21]. The proximity-induced cleavage of procaspase 9 in the assembled apoptosome results in the initiation of common effector apoptotic pathway with activation of procaspase 3 and subsequent activations of procaspases 6 and 7 [22,23].

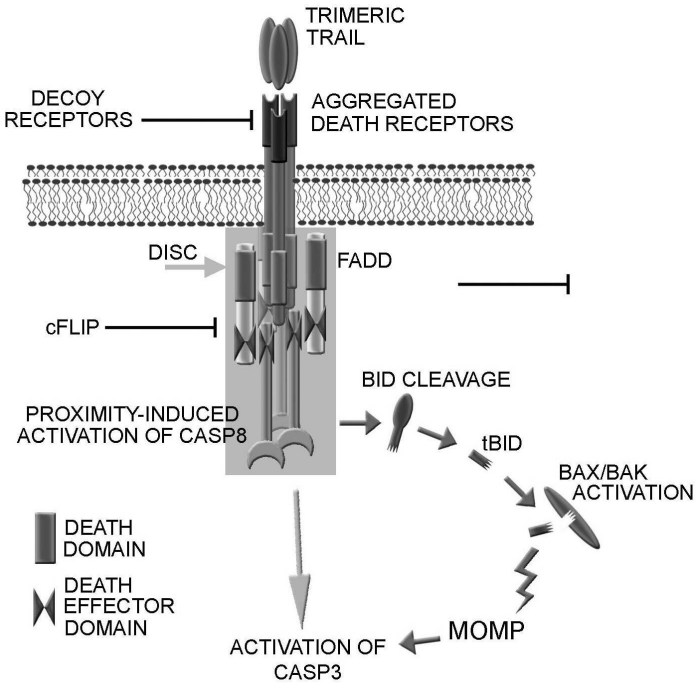
b. Receptor-mediated apoptotic pathway

Binding of a death ligand to the corresponding death receptor induces extrinsic, receptor-mediated pathway of programmed cell death. **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 [24,25]. 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 (TNFSF2, cachectin), Fas-ligand (TNFSF6, FasL, CD95 ligand, Apo1L), and TNF-related apoptosis inducing ligand (TRAIL, TNFSF10, Apo2L). Receptors for TNF family ligands are type I membrane proteins that belong to the superfamily of TNF receptors (TNF-R) [26]. Based on the ability to transduce death signal the TNF-R family can be divided into **death receptors (DR)** and **decoy receptors (DcR)**. Highly conserved **death domain (DD)** motifs, responsible for conveying death signal, are part of the intracytoplasmic portion of death receptors. Death receptors include TNF-R1 (TNFRSF1A), TNF-R2 (TNFRSF1B), Fas (TNFRSF6, Apo1, CD95), TRAIL-R1/DR4 (TNFRSF10A, Apo2), and TRAIL-R2/DR5 (TNFRSF10B) [27-30]. 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 (TNFRSF10C), and TRAIL-R4/DcR2 (TNFRSF10D). Soluble decoy receptors comprise DcR3 (TNFRSF6B), and osteoprotegerin (OPG, TNFRSF11B, TRAIL-R5) [31-34]. For decoy receptors cannot activate apoptosis they compete for specific death ligands with corresponding death receptor. Proportion of death and decoy receptors on cell surface might be critical for

the sensitivity/resistance of the cells to apoptotic stimuli triggered by death ligand.

Members of the TNF ligand family (i.e. TNFα, FasL, and TRAIL) are arranged and function as homotrimers [35,36]. A death ligand homotrimer binds to the trimer of corresponding TNF receptors. Higher-order receptor complexes might form as a result of conformational change caused by ligation-induced receptor trimerization. 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** (FLICE) via homologous **death effector domain (DED)** motifs, which results in the assembly of a multiprotein complex called **death inducing signaling complex (DISC)** [37-39]. 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.

Figure ii: Receptor-mediated apoptotic pathway (legend in text)



c. 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 (laminin, actin, cytokeratin, fordrin), and cytoplasmic membrane alterations, including membrane blebbing with apoptotic bodies forming from the surface of the shrinking cell [40-44]. 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.

The executive phase of apoptosis is carried out by cysteine proteases called **caspases** (cysteine aspartic acid-specific proteases) [45,46]. 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 two groups: enzymes that participate in apoptosis (caspases 2, 3, 6, 7, 8, 9, and 10), and enzymes that play role in cytokine processing and inflammation (caspases 1, 4, 5, 13, and 14). Apoptotic caspases can further be subdivided 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 [47-50]. 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. Various enzymes that are involved in cellular disintegration are activated by caspases, such as CAD (caspase-activated DNAse), responsible for DNA

fragmentation, or gelsolin (actin depolymerizing enzyme), responsible for cell membrane blebbing [51-56]. Caspases 3 and 7 can cleave and inactivate PARP (poly(ADP-ribose) polymerase), a DNA repair enzyme that is activated by DNA breaks and that catalyses the attachment of ADP-ribose polymers to acceptor proteins. The PARP repair function is associated with cell ATP store depletion, which may result in switch from programmed cell death, apoptosis, to energy independent cell death, necrosis [57-60].

d. 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 [61-64].

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 [62,65-67].

Besides tBid, both apoptotic cascades seem to crosstalk by means of additional molecules or pathways. Death receptors can trigger non-apoptotic signaling by recruiting adaptor molecules RIP (receptor interacting protein) or TRAF2 (TNF-receptor associated factor2). These adaptors represent upstream components of signaling pathways that lead to activation of many protein kinases and/or transcription factors.

The JNK (Jun N-terminal kinase) and NFκB (Nuclear Factor kappa B) pathways represent important signaling pathways triggered by activated death receptors. In general, **JNK pathway** exerts proapoptotic, while **NFκB pathway** prosurvival functions [68-71]. NFκB activation suppresses apoptosis induced by TRAIL

at least in part by antagonizing JNK pathway [72-76]. Translocation of NFκB transcription factors to the nucleus induces transcription of NFκB target genes, including important inhibitors of apoptosis, e.g. **IAP(s)** (inhibitor of apoptosis protein(s)) [77,78].

Apart from the above given examples of well-established extrinsic-to-intrinsic apoptotic crosstalk, there are additional important links between the apoptotic signaling pathways. p53 tumor suppressor, for example, not only binds directly to the mitochondrial membrane thereby impacting the intrinsic apoptotic pathway, but also enhances transcription of death receptors (namely TRAIL-R2/DR5), which increases susceptibility of the damaged cells to triggers of extrinsic apoptosis [79-81]. Several other important transcription factors (e.g. cMyc, cFos, cJun etc.) appear to function in a similar way [82]. Both radiotherapy and several anti-cancer agents (e.g. anthracyclin antibiotics, cisplatin, etoposide etc.) induce cell surface upregulation of death receptors indirectly, via the p53-mediated mechanism [83,84]. Hence, combined treatment of tumor cells with chemo/radiotherapy and death ligands often results in cytotoxic synergy when compared with the killing effects of cytostatics or death ligands alone [85-91].

e. 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 recruitment of adaptor molecules TRADD/FADD and thereby transduction of the apoptotic signal. **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 [92-98].

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 [99-103]. The family can be divided into antiapoptotic and proapoptotic group. The proapoptotic Bcl-2 proteins evolved as molecules corresponding to a plethora of stress stimuli from the environment or from within the cell, and their major role lies in conveying the stress stimuli into the apoptotic pathway. The proapoptotic group can further be divided into subgroup of proteins that contain in their structure only one *Bcl-2 homology domain (*Bcl-2 homology domain 3, BH3), called **BH3-only proteins**. BH3-only proteins comprise large family of proapoptotic modulators: *Bad, Bag, Bid, Bik, Bim, Bcl-xS, Puma, Noxa* etc. They act either to activate multidomain Bax and Bak members or to interfere with the antiapoptotic Bcl-2 family (see later). **Multidomain (BH-123) proteins** Bax and Bak represent the latter, effector subgroup of Bcl-2 proapoptotic molecules. Activated Bax/Bak proteins translocate from the cytoplasm to the mitochondrial outer membrane, oligomerize and by forming pores they precipitate MOMP, which leads to mitochondrial disruption and subsequent apoptosome

assembly.

Antiapoptotic members of the Bcl-2 family inactivate proapoptotic Bcl-2 proteins mainly via forming dimers with one another. The antiapoptotic Bcl-2 family consists of *Bcl-2, Mcl-1, Bcl-xL, Bcl-w, Bcl-G, Boo/Diva, Bcl-B, Bcl-G* etc. Bcl-2 protein is a key antiapoptotic regulator. It constitutively associates with both the mitochondrial and endoplasmic reticulum (ER) membranes.

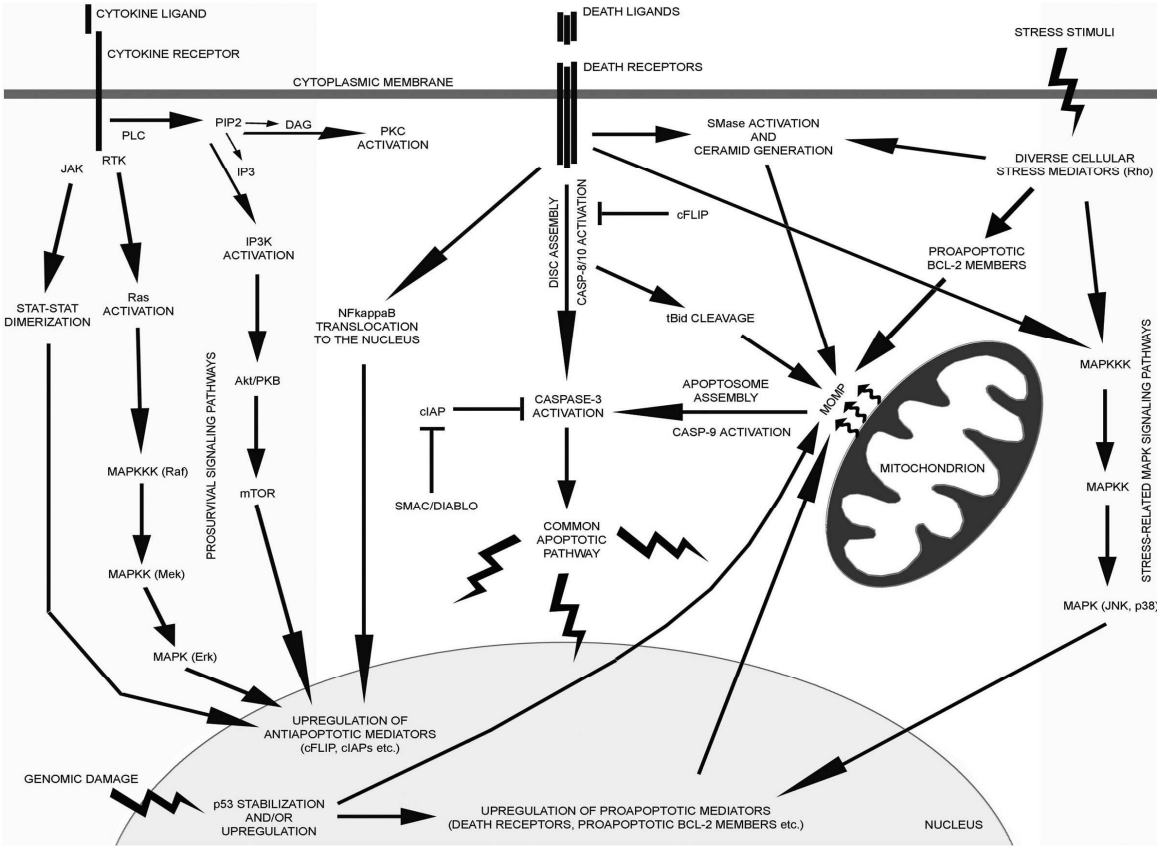
Heat shock proteins (HSP(s)) constitute powerful modulators of both the mitochondrial and the receptor-mediated apoptotic cascades. HSPs represent a network of chaperone molecules, some of which are constitutively expressed but others rapidly induced in response to a variety of stress stimuli. HSPs serve to disaggregate, refold and renature misfolded or aggregated proteins, which protects cells from several forms of death, including apoptosis [104-107]. In normal cells, the expression of most HSPs is low, but can be induced by stress due to misfolded or denatured proteins (e.g. heat shock, mutated proteins). Various members of HSPs family (Hsp70, Hsp90) are abundantly expressed in tumors, where they contribute to cancer cell survival by stabilizing diverse aberrant proteins with direct or indirect antiapoptotic activities. Hsp70 binds adaptor protein Apaf-1, which blocks the assembly of apoptosome. Hsp27 can successfully block Smac/DIABLO proapoptotic enhancer. Hsp90 takes active part in NFκB signaling pathway [108-110].

Inhibitor of apoptosis proteins (IAP(s)) 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 [111-116]. In many *in vitro* studies overexpression of IAPs correlated tightly with multi-resistant phenotype. IAPs are capable of both inhibiting the cleavage of procaspases and blocking the activated caspases. 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 [117,118].

The ubiquitin/proteasome system comprises a large proteinase complex that is responsible for the turnover of various intracellular proteins. Many of the key regulators of apoptosis are substrates of proteasome, e.g. p53, NFκB, Bcl-2 family members Bax, Bad etc. Hence, ubiquitin/proteasome belongs among indirect, yet extremely important players that interfere with various death signals [119-122].

Figure iii: Interconnected proapoptotic and antiapoptotic pathways (legend in text)



II. TRAIL

a. Introduction

It is widely accepted paradigm that in transformed, unlike normal cells, even reversible damage can trigger and carry out programmed cell death. Huge body of evidence supports the fact that a great number of anti-cancer agents act via inducing the intrinsic apoptotic pathway(s) in malignant cells. Drug resistance in cancer then frequently is associated with the intrinsic apoptosis program deregulation, in particular mutations of p53 pathway. The extrinsic death cascade, however, often remains intact in such cells, and many cytostatic-resistant primary tumor cells or cell lines remain sensitive to death-ligand induced apoptosis. In addition to that, it has been reported that stimulation of death receptor pathways might render the drug-resistant cancer cells sensitive (=they can be resensitized) to cytostatics. Thus, the combined effect of triggering both, extrinsic and intrinsic, cell death cascades appears optimal to elicit the maximal therapeutic impact, even in multi-drug-resistant tumor cells [123-127].

b. 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 [128]. TNFα superfamily proteins regulate development and function of the immune system. They are involved in induction of cytokine secretion, upregulation of adhesion molecules, activation by antigens and co-stimulatory proteins.

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).**

c. 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. TRAIL is structurally type II membrane glycoprotein, whose C-terminal extracellular domain shows clear homology to other TNF family members [35,129]. Like FasL and TNFα, the C-terminal extracellular region of TRAIL (amino acids 114-281) exhibits a homotrimeric subunit structure [36,130]. 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 [131-133]. Membrane-bound TRAIL (mTRAIL) can be cleaved by specific metalloproteinases to yield trimeric soluble form (sTRAIL).

Both membrane-bound and soluble TRAIL induces extensive apoptosis in a plethora of tumor cell lines and primary cells, including hematologic malignancies [134]. Direct anti-tumor activities of TRAIL probably represent main physiologic role of TRAIL in the context of more complex **tumor immune-surveillance** [135]. Unlike FasL, TRAIL and its receptors are widely expressed in a variety of normal tissues, which are resistant to TRAIL-triggered programmed death [136,137]. As a consequence, strong negative regulation of TRAIL-induced apoptosis can be anticipated in most normal cells [138]. 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, remaining for caspase 10 [37-39,139-142]. In addition to caspases 8 and 10, several studies have recently brought evidence for a potential role of another apical caspase, caspase 2, in TRAIL-induced signaling [143,144]. Apart from triggering apoptosis, TRAIL has also been shown to be

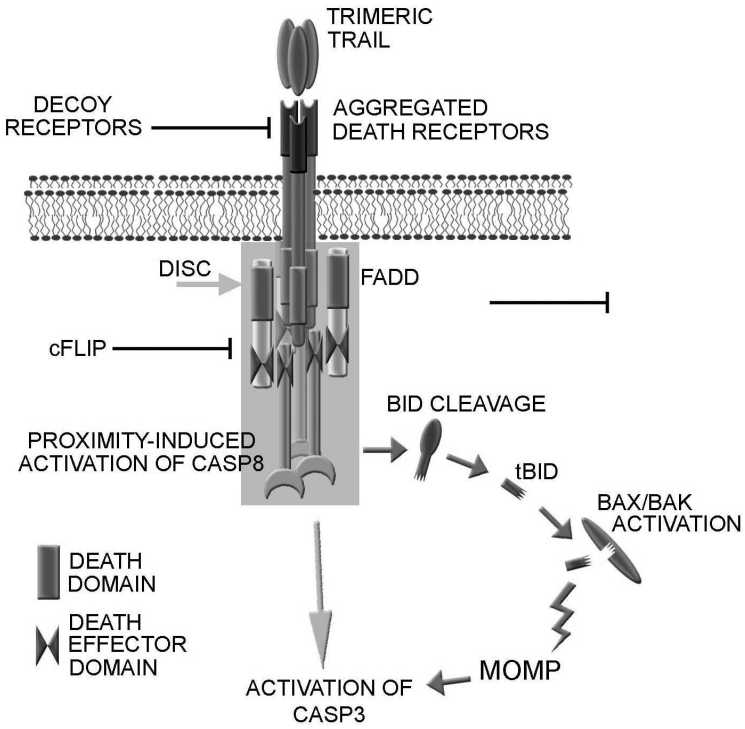
able to induce necrotic death pathway [145-147].

Consequently, much enthusiasm has been generated for TRAIL as a potential, highly specific and safe anti-cancer drug. TRAIL does not induces apoptosis of normal tissues [134,148-150]. Despite considerable data about the safety of TRAIL it remains, however, anxiety over potential hepatotoxicity of TRAIL under specific conditions [151,152]. 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 [153-161].

d. Receptors for TRAIL

TRAIL can bind to five receptors, which represents the most complex receptor system for one ligand in a human body [162,163]. Receptors for TRAIL include **death (DR) receptors** and **decoy (DcR) receptors** [27-30,33,34,164-166]. 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 [167-170].

Figure ii: Receptor-mediated apoptotic pathway (legend in text)



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. Initiator procaspase 8 (and to much lesser extent procaspase 10) is then recruited to FADD through homophilic **death effector domain (DED)** interaction, as it was described for TNFα [171-173]. 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. 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**, and **I3PK-PKB/Akt-mTOR** pathways, and mostly proapoptotic **JNK**, and **p38 stress kinase** cascades [74,164,165,174-177]. TRAIL triggers these pathways by promoting the association of a **secondary signaling complex**, subsequent to assembly of a primary, death-inducing signaling complex (DISC). The secondary complex retains the DISC components FADD and caspase-8, but recruits other molecules that are directly involved in kinase activation by TRAIL, namely **RIP1** (receptor-interacting protein 1), **TRAF2** (TNF receptor associated factor 2), and **NEMO/IKKγ** (NFκB essential modulator / inhibitor of kappa B kinase gamma) [178]. These proteins play essential roles as upstream relay-proteins conveying signals from the TRAIL-activated receptors to downstream effector molecules.

According to recently published data, RIP1 plays essential role in activating both IκB kinase (IKK, inhibitor of kappa B kinase) and JNK [179]. Activation of IKK leads to IκB phosphorylation, ubiquitination and degradation in proteasome, which induces the activation of NFκB transcription factor, its translocation to the nucleus, and subsequent transactivation of NFκB target genes. Activation of NFκB pathway usually confers strong antiapoptotic, prosurvival effects on the cells. JNK leads to activation of transcription factor AP-1, and its effects on cell survival may be proapoptotic, as well as antiapoptotic, depending on cell type and circumstances [180-183]. RIP1 has also been reported to be able to induce necrotic cell death under special conditions [184,185]. TRAF2 plays little role in TRAIL-induced IKK activation, although it is required for TRAIL-mediated JNK activation [179,185-188].

TRAIL-R3/DcR1 lacks any intracellular part, and is tethered to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor [34]. **TRAIL-R4/DcR2** has a truncated, non-functional death domain [189]. 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 [32]. Decoy receptors (including OPG) thus act as competitive inhibitors of death receptors for TRAIL ligand binding. Depletion of TRAIL available for death receptor-mediated apoptosis is, however, not the unique mechanisms by which decoy receptors (but not OPG) are capable to block apoptosis. As it was mentioned earlier, trimeric TRAIL binds three monomeric chains of TRAIL receptor. The receptor trimer can, however, constitute of a **homotrimer** or a **heterotrimer**. It has been experimentally proved that while TRAIL-R3/DcR1 prevents the assembly of the death-inducing signaling complex by titrating TRAIL within lipid rafts, TRAIL-R4/DcR2 is co-recruited with TRAIL-R2/DR5 within the DISC, where it

prevents caspase 8 activation . In addition, TRAIL-R4/DcR2 prevents TRAIL-R1/DR4 recruitment within the TRAIL-R2/DR5 DISC [190]. The level of death or decoy receptor expression doesn't, however, always correlate with susceptibility of the cells to undergo TRAIL-induced apoptosis, suggesting other mechanisms controlling death ligand homeostasis.

It has been shown that TRAIL receptors exist on cell surface in a preassembled form (as homo- or heterotrimers). Ligation of TRAIL then activates the self-associated receptor complexes. Such a pre-ligand receptor binding is possible due to pre-ligand assembly domains (PLADs) in the extracellular part of TRAIL receptors [191,192]. Hence, PLADs might represent yet another powerful homeostatic mechanism orchestrating TRAIL-induced apoptosis [193]. Last but not least, TRAIL-R4/DcR2 induced activation of prosurvival signaling pathways, namely NFκB, has been reported as additional mechanism of possible death receptor-induced apoptosis suppression [165].

Recently, two novel alternative splice variants of TRAIL have been detected in neoplastic and normal human cells. The transcripts lack either exon 3 (TRAILβ) or exons 2 and 3 (TRAILγ) [194]. The reported alternative splicing of TRAIL might thus be involved in additional fine tuning of TRAIL-induced apoptosis, which would only further underscore the complexity of the TRAIL-receptor system.

e. Cytotoxic synergy of TRAIL and anti-cancer agents

Most chemotherapeutic agents used in the treatment of leukemia/lymphoma induce the mitochondrial proapoptotic pathway [195]. 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 [196,197]. 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. 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 [83,83,86,90,198-220].

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

f. TRAIL: a novel therapeutic agent?

Convincing scientific body of evidence suggests TRAIL could prove efficient in the treatment of various neoplastic diseases, especially when used in combination with currently used anti-cancer agents and/or radiotherapy [83,86,90,199,200,200,201,201-208,208-219,221-223]. Alternatively, TRAIL could be used for *ex vivo* purging of leukemia cells before stem cell transplantation [224].

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* [134,148]. Injection of any of the above given soluble forms of TRAIL did not reveal any toxicity in rodents or non-human primates. The biologic half-live of soluble TRAIL given as an *i.v.* bolus to mice (10-15 mg/kg), rats (10 mg/kg), cynomolgus monkeys (1-50 mg/kg), and chimpanzees (1 and 5 mg/kg) was approximately 3 to 5 minutes in rodents, and approximately 23 to 31 minutes in nonhuman primates, indicating rapid elimination of TRAIL from the serum of all species studied [150]. 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.

g. Agonistic monoclonal antibodies against TRAIL death receptors, and experimental TRAIL-based gene therapy

Monoclonal antibodies against TRAIL death receptors represent an alternative to recombinant TRAIL protein. Antibodies mimic the proapoptotic activity of the natural TRAIL, and are biologically more stable [225-227]. The drawback of the monoclonal antibodies is their monospecificity, because they engage only one of the two death receptors, when compared to bispecific rhTRAIL. On the other hand, it has been shown that monoclonal antibodies might exert apoptotic function even in some TRAIL-resistant cell lines [228]. Besides the direct induction of apoptosis by F(ab)2 fragment, monoclonal antibodies exert additional substantial cytotoxic effect on the target cells by their respective Fc fragment, in particular by activating complement cascade and recruiting cytotoxic T-cells. Phase I and II clinical trials are underway evaluating pharmacokinetics, safety and anti-cancer properties of **anti-TRAIL-R1/DR4 (HGS-ETR1/Mapatumumab)** and **anti-TRAIL-R2/DR5 (HGS-ETR2/Lexatumumab)**, respectively, in a variety of malignancies, including advanced lymphoproliferative diseases [229] (http://www.hgsi.com/products/index.html).

TRAIL-based gene therapy represents another possible approach to the treatment of diverse malignancies [230-233]. TRAIL gene transfer strategy might target either malignant cells themselves, or potential non-malignant effector cells that would specifically kill tumor cells by producing secreted or membrane-bound forms of TRAIL. Extensive search is under way both for suitable vector type and for appropriate "carrier" cells that would specifically be attracted by and migrate to the tumor cells [234-243].

h. 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. **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), and the frequently observed loss of chromosome 8p21-22 in NHLs might be relevant to the death receptors [244]. 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 [245]. In other words, malfunction mutations of TRAIL death receptors might act in a dominant negative manner toward their respective normal (wild type) alleles. However, no mutation or loss of heterozygosity was found in chronic myeloid leukemia (CML) and myelodysplastic syndrome (MDS) patients, respectively [246]. **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 [247-250]. 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 [31,251-253].

Disruption of DISC components, namely downregulation, loss-of-function mutation or increased turnover of apical caspase 8, and deregulation of adaptor protein FADD, respectively, have been reported responsible for constitutive or acquired TRAIL resistance of tumor cells [140,172,254-260].

Overexpression of cFLIP represents one of the best-characterized mechanisms associated with TRAIL and FasL resistant phenotypes [95,198,261-266]. Thus,

targeting cFLIP expression might represent useful approach in restoring TRAIL sensitivity [93,267,268].

Increased expression of antiapoptotic family proteins, **IAPs** (in particular **XIAP**) and **HSPs** (namely **Hsp90**), conferred TRAIL resistance to a wide range of malignant cells [110,201,221,269-271]. 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 [272-274].

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 and PI3K-PKB/Akt-mTOR** (phosphoinositol-3-kinase-protein kinase B/Akt-mammalian target of rapamycine), deregulation of stress-related kinase pathways, **JNK** and **p38**, or disruption of other essential kinases, e.g. **PKC** (protein kinase C), **CKII** (casein kinase II) etc. [72,77,82,174,185,185,198,198,200,275-286]. Susceptibility to TRAIL was correlated with the expression of key cellular transcription factors and oncogenes, namely Myc, Jun and others [98,287-289]. A plethora 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 [290-301].

III. SUMMARY

Advanced tumors, including leukemia, represent heterogeneous cell populations evolved from original malignant clones. Chemotherapy of leukemia is often associated with selection of drug-resistant cells followed by progression/relapse of the disease. Implementation of molecules that specifically target leukemia cells with minimal toxicity to normal tissues might significantly improve outcome of leukemia treatment. TRAIL belongs to the tumor necrosis factor (TNF) ligand family of cytokines. TRAIL triggers apoptosis in target cells via the receptor-mediated apoptotic pathway. Receptors for TRAIL can be divided into death receptors, TRAIL-R1/DR4 , TRAIL-R2/DR5, and decoy receptors, TRAIL-R3/DcR1, TRAIL-R4/DcR2, osteoprotegerin/OPG/TRAIL-R5, based on their ability to transduce apoptotic signal. While normal tissues, including hematopoietic progenitor cells, are resistant to TRAIL-induced apoptosis, TRAIL induces programmed death in many tumor cell lines and primary cells. Various malignant cell lines and primary tumor cells, however, show resistance to TRAIL-induced apoptosis. TRAIL-resistance could represent important limitation for the potential TRAIL anti-tumor therapy. Combined *in vitro* application of TRAIL with other anti-cancer agents often increased sensitivity or overcame resistance of the tumor cells to the given chemotherapeutics or to TRAIL-induced apoptosis. The combination of TRAIL and cytotoxic agents improved therapeutic outcome compared to TRAIL or cytotoxic agents alone in several preclinical studies in tumor xenotransplanted immunodeficient mice models. Despite the work that has been accomplished the therapeutic potential of interactions between TRAIL and a variety of established or experimental chemotherapeutical agents are still incompletely understood. Several molecular mechanisms responsible for TRAIL

resistance have been described up to now. Most of the studies, however, focused on constitutive TRAIL resistance in solid tumors. Molecular mechanisms responsible for acquired TRAIL resistance in hematological malignancies remain to the large extent elusive.

EXPERIMENTAL STUDY

1. 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[302]

2. MATERIALS AND METHODS

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

The extracellular domain of human TRAIL gene (coding for amino acids 95–281) was isolated by RT-PCR from HPB T cell line, sequence-verified and subcloned into E. coli expression plasmid containing N-terminal His6 tag. 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 purity of the recombinant His-TRAIL (Killer TRAIL, Alexis, San Diego, CA, USA) was determined by SDS-PAGE electrophoresis.

Cells were cultured in Iscove's modified Dulbecco's medium (Life Technologies, MD, USA) in the presence of 10% fetal bovine serum (FBS) in 37°C humidified atmosphere with 5% CO_2. The expression of TRAIL receptors was measured by flow cytometry (FACS Calibur; Becton Dickinson, CA, USA) using anti-TRAIL receptor antibodies (R&D systems, MN, USA) and corresponding secondary antibody (Jackson Immuno Research Laboratories, Inc., USA). TRAIL death receptors were blocked with rabbit polyclonal antibodies to TRAIL-R1/DR4 (B5/B4), TRAIL-R2/DR5 (5CII) (Apronex Biotechnologies, Czech Republic). TRAIL decoy receptors were blocked with goat polyclonal antibodies (R&D systems, MN, USA).

Apoptosis was analyzed by flow cytometry (FACS Calibur) using Annexin-V-FITC staining (Apronex Biotechnologies, Czech Republic). The pharmacological blockage of apical caspases 8 and 10 was achieved by preincubation of the cells with caspase specific inhibitors Z-IETD-FMK, Z-AEVD-FMK, and Z-VAD-FMK at concentrations ranging from 0,64 to 80μM (R&D systems, MN, USA).

Establishment of TRAIL-resistant and cytostatic-resistant HL60 cells

Proportion of TRAIL resistant cells in the WT HL60 population was detected using limiting dilutions. Cells were distributed onto 96-well plates (10 to 500 cells per well) in medium containing TRAIL (20, 200, or 2000ng/mL). On day 30, the wells with proliferating cells were counted and the proportion of TRAIL-resistant cells was calculated.

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.

TRAIL-resistant HL60 cell *clones* were established as follows: HL60 cells were diluted (5 cells per 1 ml) and distributed onto 96-well plates (100μl per well). Wells that contained a single HL60 cell were subject to further manipulation. In two weeks the cells of each well were split into 8 wells and exposed to increasing concentrations of TRAIL (4, 8, 15, 30, 50, 100, 200 ng/mL). The sensitivity to TRAIL was determined and TRAIL unexposed cells from TRAIL-resistant clones were subject to further analysis.

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

RNA was phenol/chlorophorm extracted from 10^6 cells homogenized in RNA-blue (Top-Bio s.r.o., Czech Republic). RNA was reverse transcribed into cDNA using

RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). Real-time PCR analysis was performed using IQ™5 multicolor real-time PCR detection system (Bio-Rad, USA). The results were normalized to actin mRNA expression. Interassay variability showed that differences between actin expressions from each experiment were less than 0,5 cycles.

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

For induction of apoptosis, one million cells were incubated either with TRAIL 200ng/ml or TNFα 100ng/ml for 0, 1, 2, and 4 hours. Caspase 8 and 10 activation was measured by Caspase8/FLICE and Caspase10/FLICE fluorometric assays (R&D systems, MN, USA) according to the manufacturer's protocol. Briefly, cells were washed with PBS, centrifuged and lysed in cell lysis buffer. After the addition of DTT (1mM final), 2x reaction buffer, and caspase specific fluorometric substance (IETD-AFC or AEVD-AFC) the lysates were incubated at 37 °C. The fluorescence intensity was measured by Plate CHAMELEON fluorescence reader (Hidex, Finland) after the 90 minutes incubation period.

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. Briefly, nuclear lysates were isolated at time 0, 20, 40, 60, 90, 120 and 180 minutes after the exposure of cells to TRAIL (200ng/ml). Positive control for NFκB RelA/p65 translocation assay, Jurkat nuclear lysate (Active Motif,

Belgium), was used to generate standard curve. The relative concentrations, relative units per milligram of total lysate protein, of HL60 cell lysates were extrapolated from the standard curve. The optical density of Jurkat nuclear lysate 5μg/ml was assigned the value of 100 relative units.

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. P1, P2, or WT HL60 cells (10 million in 300uL PBS) were injected into tail veins of the irradiated animals on day 1. Three groups of mice, six animals in each group, were xenotransplanted with WT HL60 cells, two cell lines of P1, and two cell lines of P2, respectively. The mice were daily observed and were sacrificed when developed hind-leg paralysis, or appeared severely sick. The cadavers were examined for macroscopic tumors. 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). HL60 cell lines re-established from the leukemic mice were further analyzed.

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) were added to the “leukemia units”, in attempt to eradicate HL60 cells. Two 96-well plates were used for

each combination and/or concentration, thus representing one treatment strategy. Two approaches were taken to measure and compare the cytotoxic effects of different treatment strategies on the corresponding leukemia units. 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).

3. RESULTS

a. Characterization of TRAIL-induced apoptosis of HL60 cells

HL60 promyelocytic leukemia cell line, WT HL60 cells, is highly sensitive to TRAIL-induced apoptosis (Fig.1a). Preincubation of the cells with anti-TRAIL blocking antibody completely abrogated TRAIL-induced apoptosis (Fig.1b).

Figure 1

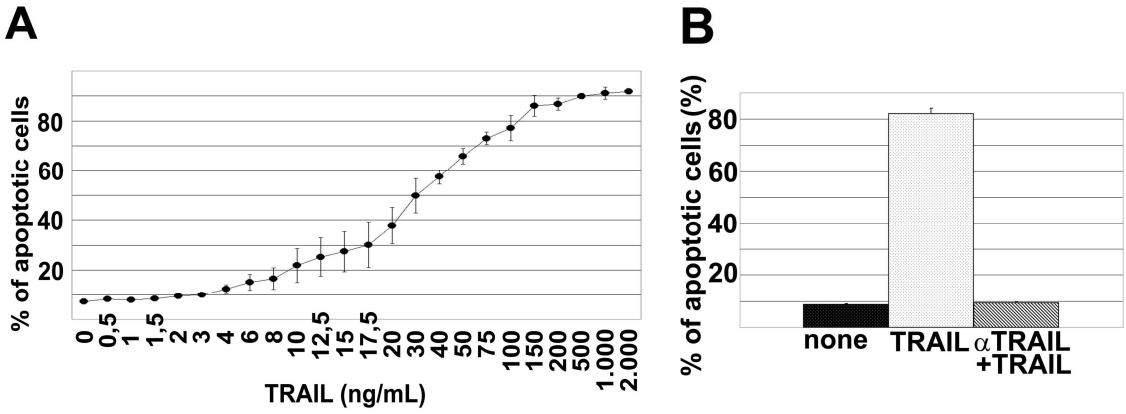


Fig.1 (A) TRAIL induces apoptosis in WT HL60 cells. HL60 cells were exposed to the increasing doses of TRAIL (0,5-2000ng/mL). (B) Blockage of TRAIL with TRAIL-specific monoclonal antibody completely abrogated TRAIL-induced apoptosis of HL60 cells. Anti-TRAIL monoclonal antibody (50μg/ml) was added to the cells 1 hour before exposure to TRAIL (200ng/ml). Level of apoptosis was analyzed by flow cytometry 24 hours after addition of TRAIL using FITC-conjugated Annexin-V assay. Data presented are means ± standard deviations of values of three independent experiments.

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 2

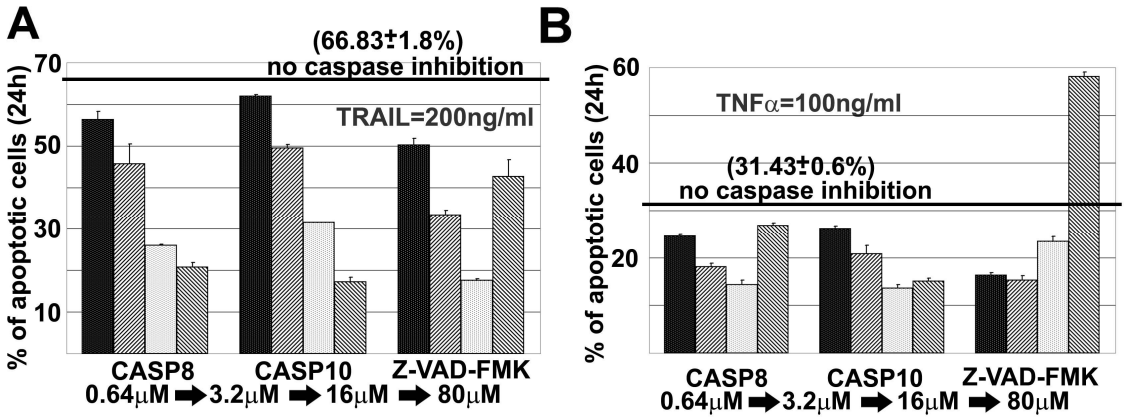


Fig.2 Caspase 8 inhibitor Z-IETD, caspase 10 inhibitor Z-AEVD and pan-caspase inhibitor Z-VAD-FMK suppressed, but did not completely abrogate, TRAIL-induced (A) or TNFα-induced (B) apoptosis of HL60 cells. Z-VAD-FMK at high concentrations increased numbers of TRAIL or TNFα-induced HL60 apoptotic cells 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).

Incubation of HL60 cells with TRAIL for at least one hour seems critical for appropriate apoptosis induction, as shorter incubations significantly reduced, and longer incubations showed no difference in the overall HL60 apoptotic cell numbers (Fig.3).

Figure 3

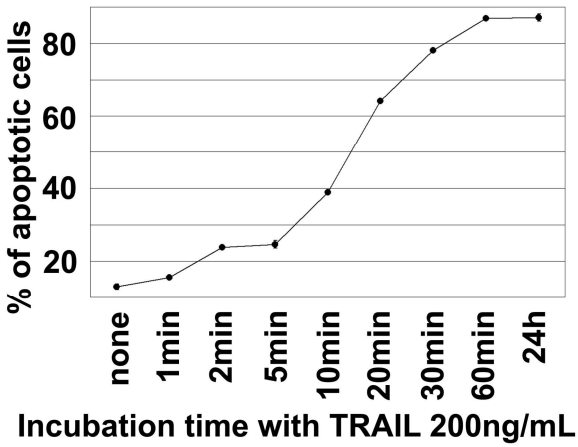


Fig.3 Exposure to TRAIL for at least one hour is critical for triggering proper apoptotic response in WT HL60 cells. The cells were incubated with TRAIL (200ng/mL) for different time intervals (from 1 minute to 24 hours). At the end of each interval the cells were centrifuged, washed and resuspended in medium without TRAIL. Level of apoptosis was analyzed by flow cytometry 24 hours after addition of TRAIL using FITC-conjugated Annexin-V assay. Data presented are means ± standard deviations of values of three independent experiments.

Limiting dilutions were performed to estimate the frequency of TRAIL-resistant cells in the WT HL60 cell line (Table 1). By using prolonged exposure of WT HL60 cells to TRAIL, we selected TRAIL-resistant HL60 cell lines (n=20).

Table 1

Number of cells per well	Number of wells with proliferating cells	Number of T-R cells
TRAIL 20ng/mL		
3,3	20/192	1/32
5	101/480	1/24
		MEAN= 1/28
TRAIL 200ng/mL		
98	8/192	1/2352
57	15/480	1/1824
		MEAN= 1/2088
TRAIL 2000ng/mL		
2159	26/206	1/17105
2549	58/480	1/21095
		MEAN= 1/19100

Tab.1 Number of TRAIL-resistant cells in WT HL60 cell line highly depends on the concentration of TRAIL. Cells were distributed into 96-well plates in medium containing Trail (20, 200, or 2000ng/mL). On day 28 wells were examined under light microscope, and total number of wells with living cells was recorded. The number of resistant cells was subsequently calculated. Table 1 shows that with increasing concentration of TRAIL, the number of TRAIL-resistant cells steadily decreases in WT HL60 cell line.

Antibody-mediated blockage of WT HL60 death receptor TRAIL-R1/DR4 more potently suppressed TRAIL-induced apoptosis than blockage of TRAIL-R2/DR5 (Fig.4).

Figure 4

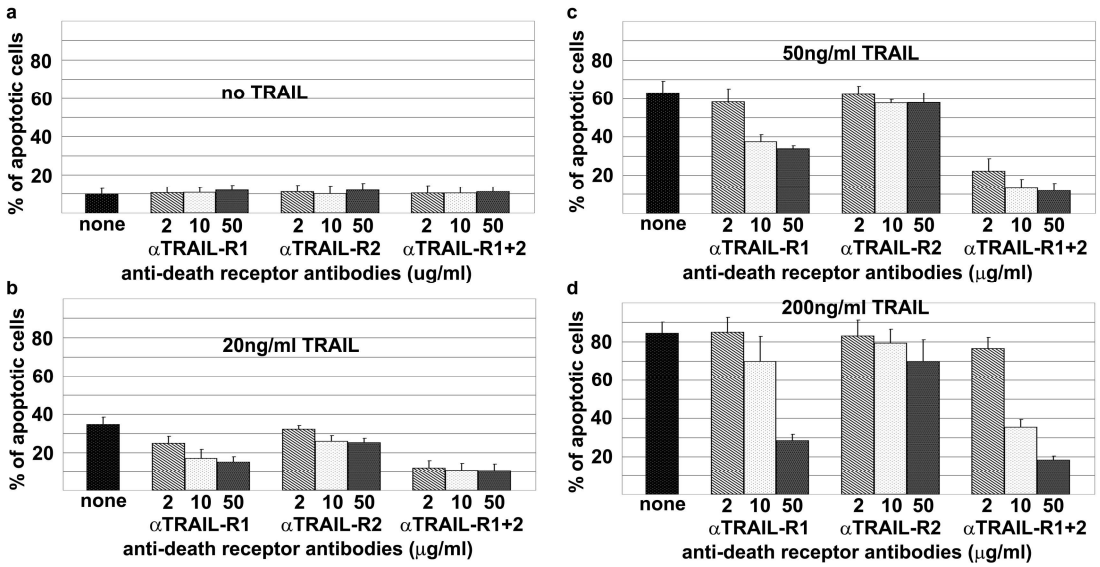


Fig.4 TRAIL-R1/DR4 transduces TRAIL apoptotic signal more potently than TRAIL-R2/DR5 in WT HL60 cells. The cells were pre-incubated with blocking antibodies against death receptor TRAIL-R1/DR4 (αTRAIL-R1), TRAIL-R2 (αTRAIL-R2), or both receptors (αTRAIL-R1+2) before exposure to TRAIL (20, 50, 200ng/mL). 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.

Antibody mediated blockage of WT HL60 decoy receptors TRAIL-R3/DcR1 and TRAIL-R4/DcR2 led to a moderate increase of apoptotic cell percentage, but the difference did not reach statistical significance (Fig.5).

Figure 5

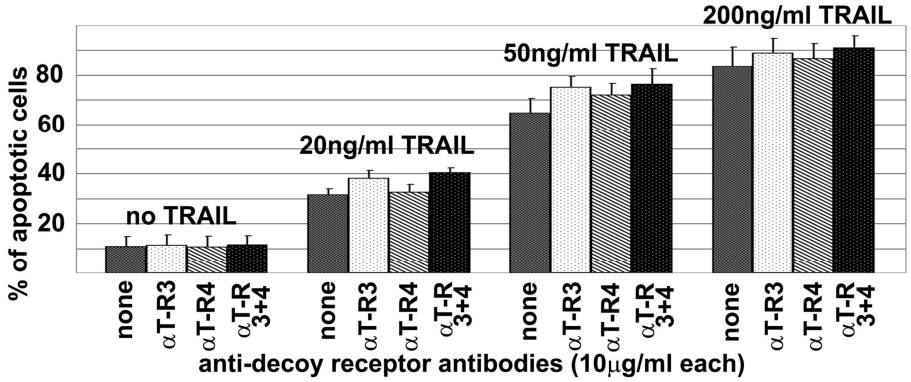


Fig.5 The increase of TRAIL-induced apoptosis in WT HL60 cells after the blockage of decoy receptors for TRAIL is statistically insignificant. The cells were pre-incubated with blocking antibodies against decoy receptor TRAIL-R3 (αTRAIL-R3), TRAIL-R4 (αTRAIL-R4), or both receptors (αTRAIL-R3+4) for 1 hour 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.

Antibody mediated blockage of decoy receptors of TRAIL-resistant HL60 cells did not influence TRAIL-induced apoptosis (data not shown). Recombinant human osteoprotegerin (rhOPG) abrogated TRAIL-induced apoptosis, and the effect of OPG could be blocked by anti-OPG antibody (Fig.6). Treatment of TRAIL-resistant HL60 cells with anti-OPG, however, did not influence the level of TRAIL-induced apoptosis (data not shown).

Figure 6

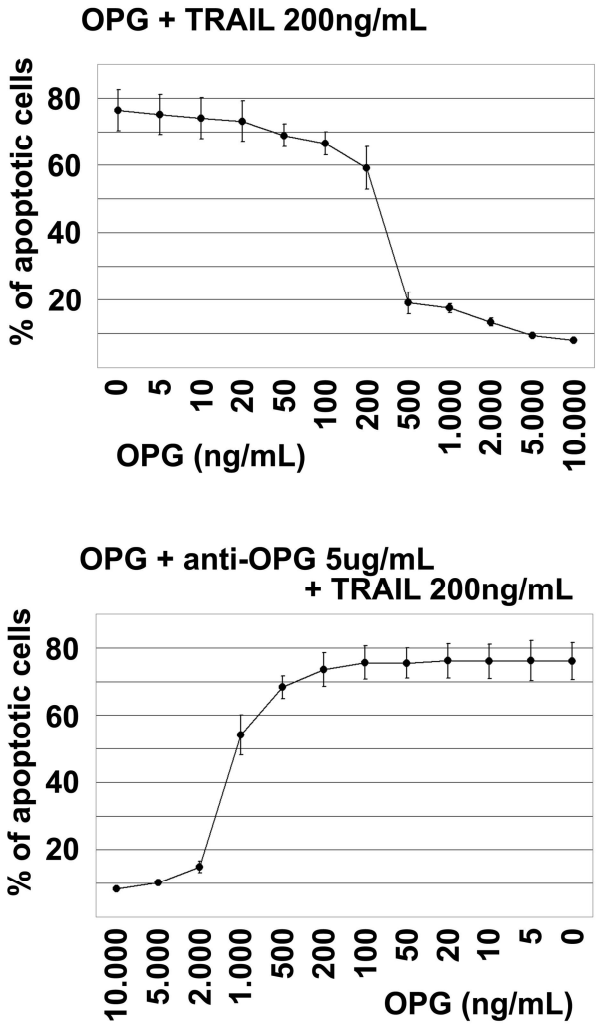


Fig.6 Osteoprotegerin (OPG) rescues WT HL60 cells from TRAIL-induced apoptosis. The cells were pre-incubated with increasing concentrations of OPG (5-10.000ng/mL) for 1 hour before adding TRAIL (200ng/mL) (Fig.6a). The cells were pre-incubated with decreasing doses of OPG (10.000-5ng/mL) together with anti-OPG antibody (5μg/mL) before adding TRAIL (200ng/mL). Level of apoptosis was analyzed by flow cytometry 24 hours after addition of TRAIL using FITC-conjugated Annexin-V assay. Data presented are means ± standard deviations of values of three independent experiments.

WT HL60 cells express TRAIL mRNA. Treatment of WT HL60 cells with anti-TRAIL blocking antibody significantly reduced level of spontaneous apoptosis (Fig.7).

Figure 7

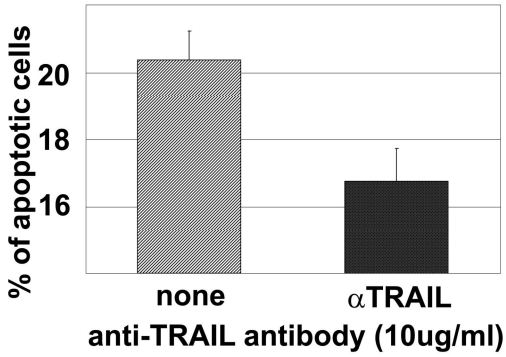


Fig.7 Endogenous TRAIL induces apoptosis in WT HL60 cells. The cells were incubated with blocking anti-TRAIL antibody for 24 hours. Level of apoptosis was analyzed by flow cytometry 24 hours after addition of anti-TRAIL antibody using FITC-conjugated Annexin-V assay. Columns represent means, and error bars standard deviations of values of three independent experiments.

In conclusion, death receptor TRAIL-R1/DR4 more potently transduces apoptotic signal than TRAIL-R2/DR5 in response to TRAIL. Neither decoy receptors, nor OPG play a role in mediating the acquired TRAIL resistance of HL60 cells after prolonged exposure of the cells to TRAIL. Endogenous TRAIL appears to play a moderate, yet statistically significant role in negative regulation of WT HL60 cell growth.

b. 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 (Fig.8, Fig.9).

Figure 8

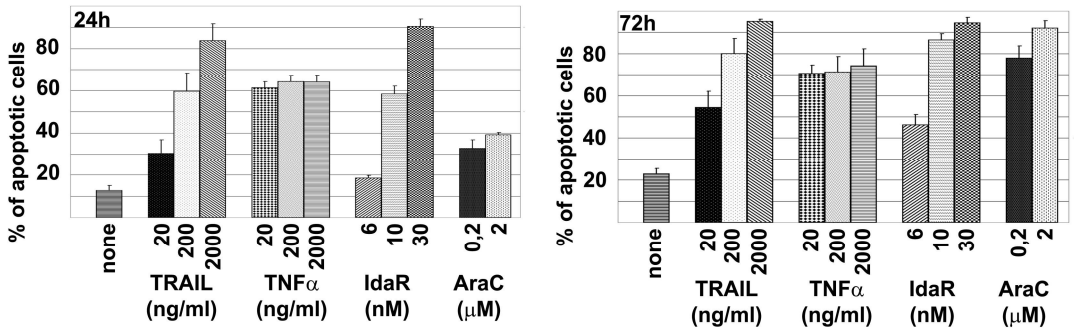


Fig.8 TNFα, TRAIL, idarubicin, and cytarabine induce apoptosis in HL60 WT cells. The level of apoptosis was measured by flow cytometry using FITC-conjugated Annexin-V assay 24 (a) and 72 (b) hours after addition of the cytotoxic drugs at different concentrations. Columns represent means, and error bars standard deviations of values of three independent experiments.

Figure 9

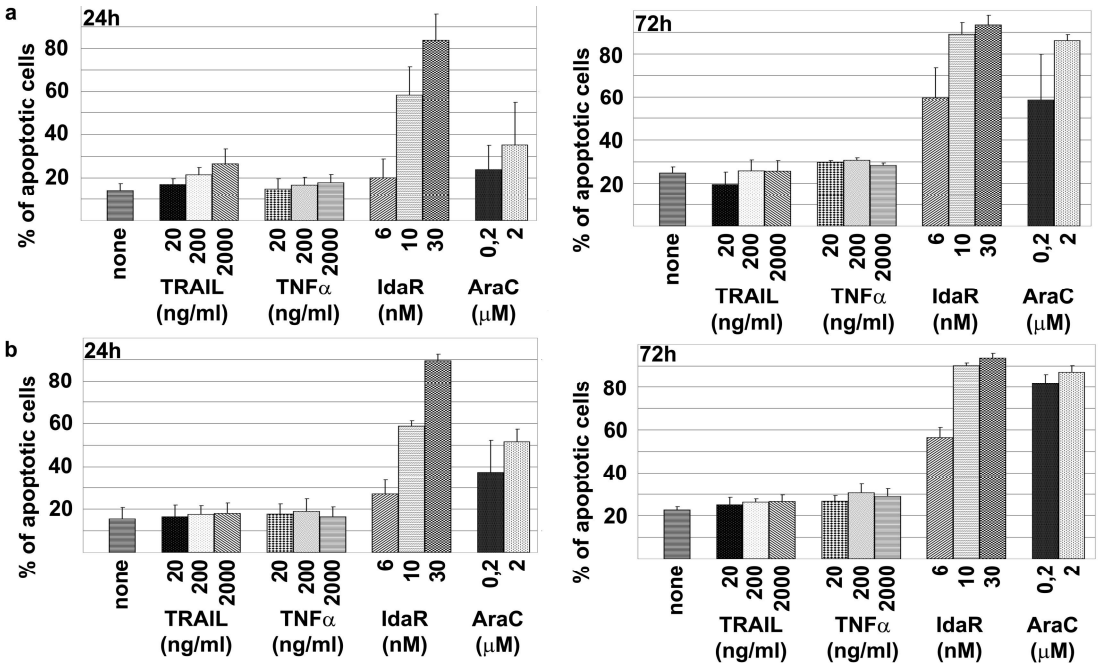


Fig.9 P1 TRAIL-resistant HL60 cells (a), and P2 TRAIL-resistant HL60 cells (b) are resistant to TNFα, but remain sensitive to idarubicin (IdaR) and cytarabine (AraC). The level of apoptosis was measured by flow cytometry using FITC-conjugated Annexin-V assay 24 (a, c), and 72 (b, d) hours after addition of different concentrations of the cytotoxic drugs. Columns represent means, and error bars standard deviations of values of three independent experiments.

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 (Figure 8, Figure 9). HL60 cells, despite expressing cell surface Fas receptor, are known to be resistant to FasLigand-induced apoptosis [302]. 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.

c. 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 (Fig.10). Acquired resistance to cytostatics was not associated with significant alterations of receptor-mediated apoptosis in HL60 cells.

Figure 10

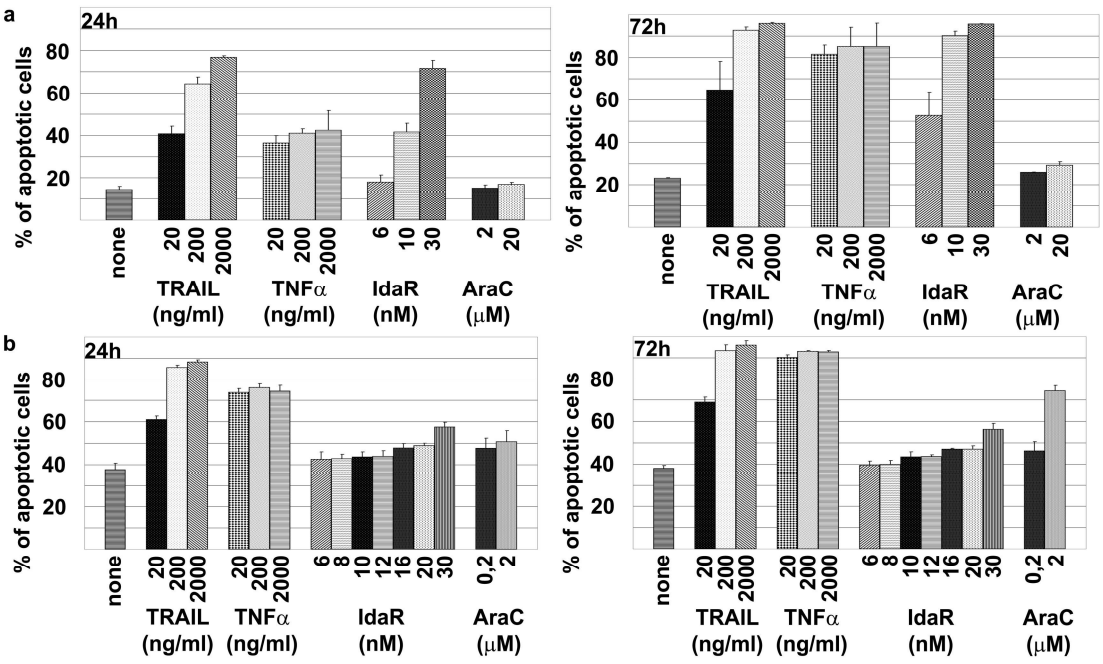


Fig.10 Cytarabine (AraC)-resistant (a), and idarubicin (IdaR)-resistant (b) HL60 cells remain sensitive to TRAIL and TNFα. The level of apoptosis was measured by flow cytometry using FITC-conjugated Annexin-V assay 24 (a, c), and 72 (b, d) hours after addition of different concentrations of the cytotoxic drugs. Columns represent means, and error bars standard deviations of values of three independent experiments.

d. 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.11, Fig.12). 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.11). 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.11, Fig.12).

Figure 11

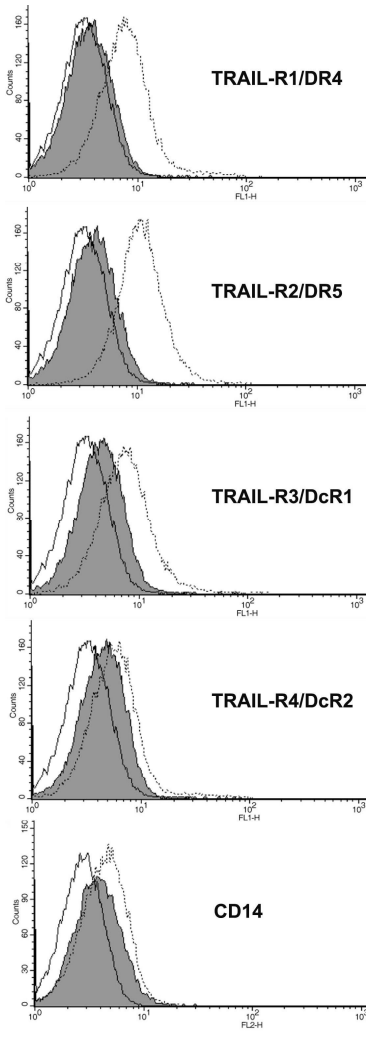


Fig.11 Cell surface expression of TRAIL receptors and CD14 in TRAIL-resistant P1 HL60 cells. TRAIL death receptors, and decoy receptor 1 (TRAIL-R1, 2, 3) are downregulated, while expression of decoy receptor 2 (TRAIL-R4) does not change in a significant way in P1 compared to WT HL60 cells. Cells were 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 (Fig.12). Neither TRAIL nor OPG were detectable by flow cytometry on the cell surface of HL60 cells (data not shown).

Figure 12

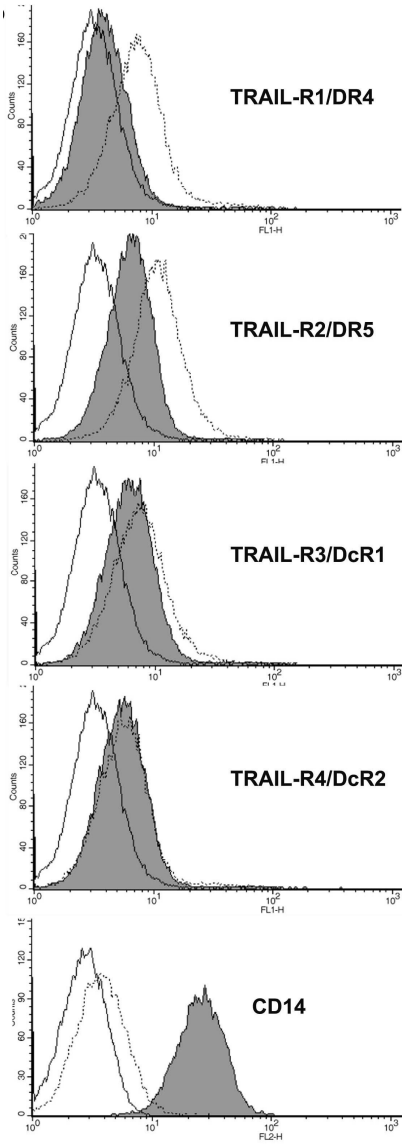


Fig.12 Cell surface expression of TRAIL receptors and CD14 in TRAIL-resistant P2 HL60 cells. TRAIL death receptors (TRAIL-R1, 2) are downregulated, while expressions of decoy receptors (TRAIL-R3, 4) do not change in a significant way in P2 compared to WT HL60 cells. Cells were stained with goat-anti-human-TRAIL-receptor 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.

On mRNA level, P1 cells showed significant upregulation of TRAIL-R4/DcR2 (2.5-fold) compared to WT HL60 cells. Interestingly, the TRAIL-R1/DR4, TRAIL-R2/DR5 and TRAIL-R3/DcR1 mRNA of P1 cells did not significantly change, which did not correlate with the decrease of cell surface expression of these receptors (compare Fig. 12 and 13). P2 cells showed significant downregulation of TRAIL-R2/DR5 (3-fold), TRAIL-R3/DcR1 (14.3-fold), and TRAIL (11.1-fold) compared to WT HL60 cells. Interestingly, the mRNA expression of osteoprotegerin (OPG) in P2 HL60 cells was not detectable by one round of real-time RT-PCR (Fig.13).

Figure 13

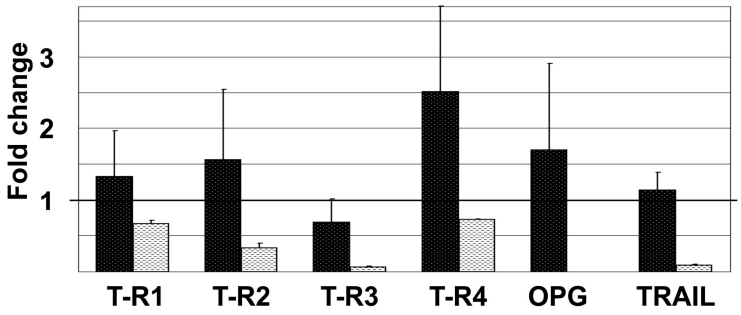


Fig.13 P2 HL60 cells lack expression of OPG. Real-time RT-PCR was carried out on WT, P1, and P2 HL60 cells. Figure 13 shows relative amounts of mRNA in P1 (dark columns), and P2 (light columns) cells compared to relative amount of mRNA in WT HL60 cells (=1). WT HL60 cells, and two cell lines from each TRAIL-resistant phenotype were analyzed. Gene expressions of TRAIL, TRAIL-R1, 2, 3, 4, and OPG were analyzed. Columns represent means, and error bars standard deviations of values of four independent experiments (two experiments for each cell lines).

All TRAIL-resistant HL60 cell lines were resistant to TNFα-induced apoptosis. Here we show that the cell surface expression of receptors for TNFα was significantly downregulated in these cell populations (Fig.14).

Figure 14

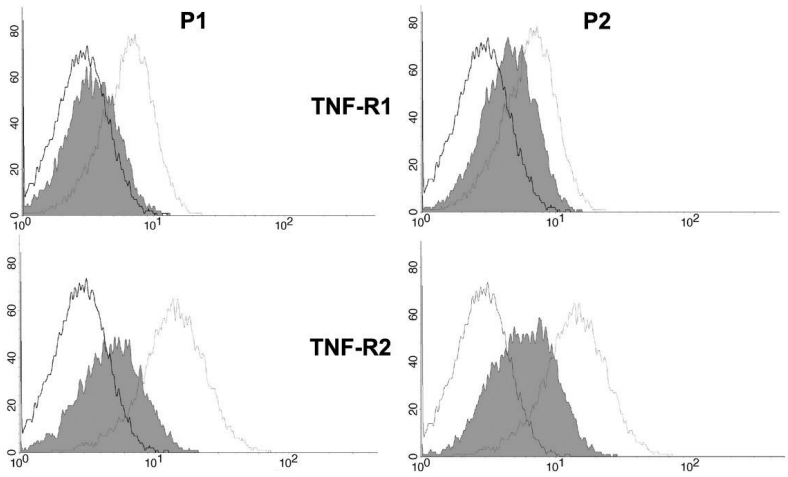


Fig.14 Cell surface expression of receptors for TNFα is significantly downregulated in TRAIL resistant HL60 cells of both phenotypes (P1 and P2= full histograms) compared to WT HL60 cells (=continuous open histograms). Cells were stained with goat-anti-human TNFα-receptor polyclonal antibody, and subsequently 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.

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

The blockage of apical caspases, caspase 8 and 10, significantly inhibited the proapoptotic effect of TRAIL and TNFα. To test the possibility that deregulated apical caspase activation caused resistance of TRAIL resistant cells to TRAIL and TNFα, we measured the activity of caspase 8 and 10 after the TRAIL and TNFα stimulation of WT HL60 cells, P1, and P2 TRAIL resistant lines. 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 (Figure 15a, b). 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 (Figure 15c, d). 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.

Figure 15

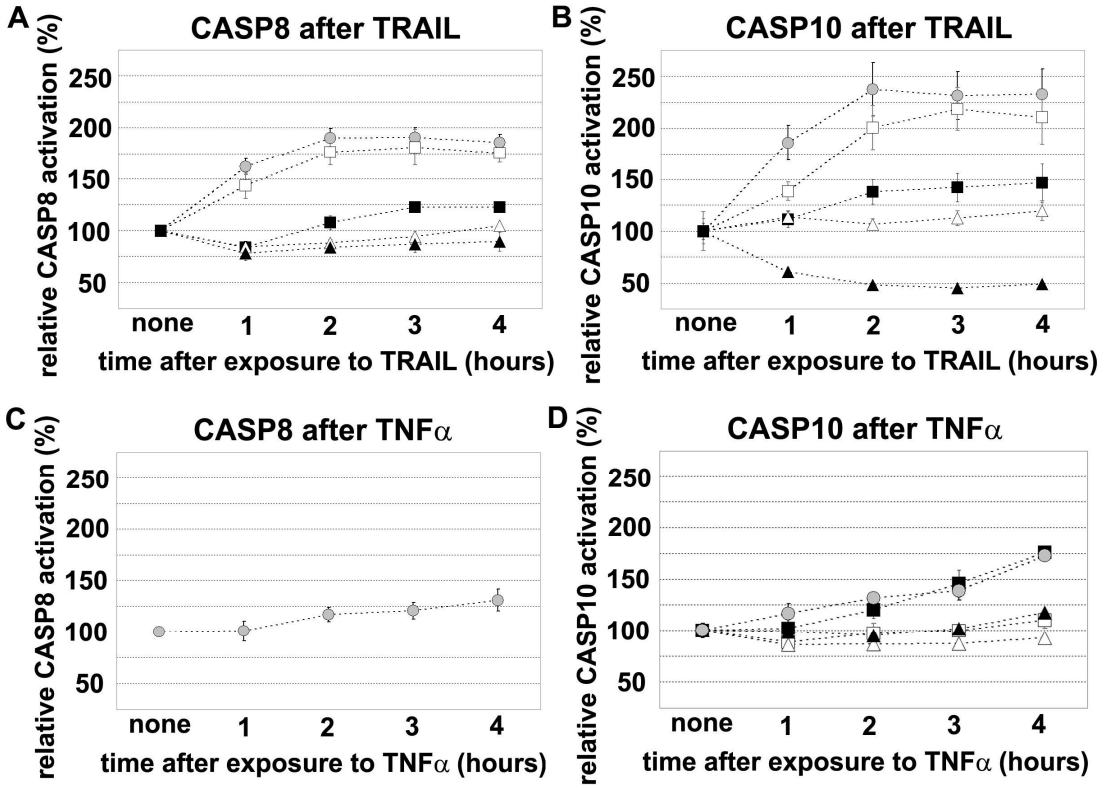


Fig.15 (A, B) TRAIL-induced caspase 8 and 10 activation is decreased in both TRAIL resistant HL60 phenotypes compared to wild type HL60 cells (P1cells= full and open triangle marks, P2 cells= full and open square marks, WT HL60 cells= full rhombus mark). (D) Similarly, caspase 10 activation is diminished after the challenge with TNFα in TRAIL resistant HL60 cells compared to WT HL-60 cells. (C) Interestingly minimal caspase 8 activation was detected in WT HL-60 cells during first 4 hours after exposure to TNFα. Results are expressed as percent increase of relative caspase activity. 100% corresponds to relative caspase activity of unstimulated cells. Results represent means ± SD of triplicate experiments.

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

Up to now, TRAIL resistance of tumor cells has been associated with changed expression of various important apoptosis regulators. 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. P1 cells showed significant mRNA upregulation of several antiapoptotic proteins: cIAP2 (9.5-fold), MCL1 (4.9-fold), cIAP1 (4.7-fold), XIAP (4.1-fold), BAG (4.8-fold), BCL2 (3.4-fold), BCL-W (3.1-fold), and BCL-XL (2.3-fold), as well as prominent upregulation of a number of proapoptotic molecules as BID (7-fold), BAD (4.8-fold), BIM-L (4.7-fold), CASP10 (3.8-fold), BIK (3.3-fold), BAK (3-fold) or SMAC (2.5-fold). P2 cells had increased mRNA expression of the proapoptotic Bcl-2 family members BAD (6.6-fold), BID (5.7-fold), BIK (2.1-fold), and downregulated expression of FADD (4.5-fold), an important adaptor molecule for TRAIL receptor transduction of apoptotic signal, together with several antiapoptotic regulators cIAP2 (5.9-fold), MCL1 (2.2-fold), and cFLIP-L (2.1-fold). None of the measured antiapoptotic regulator mRNA was more than two fold increased in P2 cells (Figure 14). These data suggest that two TRAIL resistant HL60 phenotypes, P1 and P2, have distinct mechanisms of resistance. 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 compared to WT HL60 cells were mostly proapoptotic. However, it can be deemed that marked downregulation of FADD might synergize with downregulation of TRAIL (and TNFα) death receptors, and these changes

together might result in the complete abrogation of TRAIL (and TNFα)-induced apoptotic signal propagation.

Figure 16

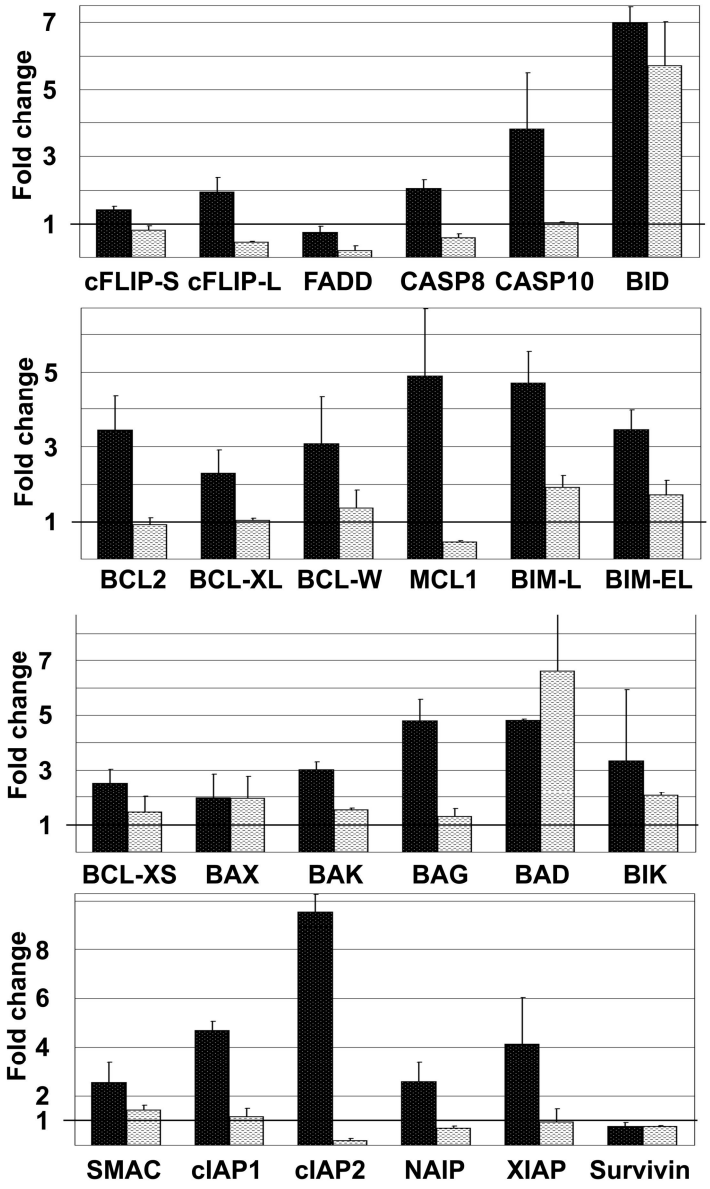


Fig.16 The expression of several essential apoptosis regulators is altered in P1, and P2 compared to WT HL60 cells. Real-time RT-PCR analysis was carried out on WT, P1, and P2 HL60 cells. Figure 14 shows relative amounts of mRNA in P1 (dark columns), and P2 (light columns) cells compared to relative 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).

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

Translocation of NFκB to the nucleus and activation of NFκB target gene expression represents an important prosurvival mechanism of malignant cells. Aberrant NFκB activation and nuclear translocation has been associated with a wide range of tumors [303-308]. TRAIL induces NFκB nuclear translocation as a result of non-apoptotic signaling pathways activation. NFκB nuclear translocation can be mediated not only by TRAIL death receptors, but also by decoy receptor 2 (TRAIL-R4). RelA/p65 NFκB member acts as a survival factor by downregulating expression of TRAIL death receptors, and upregulating expression of BCL-XL or IAP family members cIAP1, cIAP2. To analyze whether the detected upregulation of Bcl-XL and IAP family members, and downregulation of TRAIL death receptors in TRAIL-resistant cells is associated with changes in NFκB activation, we measured NFκB member RelA/p65 translocation to the nucleus after exposure to TRAIL. WT HL60 cells and two TRAIL-resistant cell lines from each TRAIL-resistant phenotype were analyzed.

The nuclear RelA/p65 level was similar in non-stimulated WT (OD = 0.148 ± 0.022) and TRAIL-resistant (OD = 0.160 ± 0.014) HL60 cells. The increase of nuclear concentration of RelA/p65 in response to TRAIL was detectable from 20 minutes after exposure of the cells to TRAIL. WT HL60 cells showed earlier onset and significantly higher peak of RelA/p65 translocation level compared to TRAIL-resistant cells of either phenotype. The peak of nuclear RelA/p65 concentration was delayed in P2 cells compared to both P1 and WT HL60 cells (Fig.17).

Figure 17

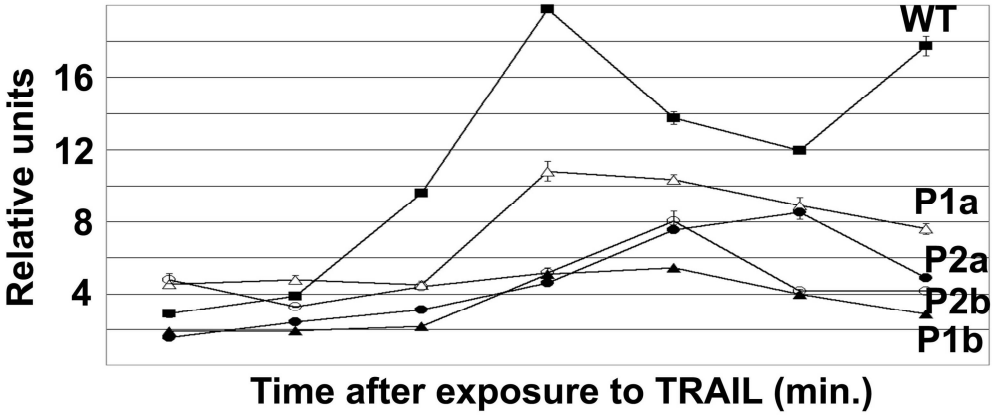


Fig.17 NFκB signaling is suppressed in TRAIL-resistant compared to WT HL60 cells. Cells were lysed 20, 40, 60, 90, 120, and 180 minutes after exposure to TRAIL (200ng/mL), and nuclear translocations of NFκB member RelA/p65, were analyzed by ELISA-based NFκB translocation assay. We observed significant decrease of the NFκB translocation peaks in cells of both TRAIL-resistant HL60 phenotypes (P1, P2) compared to WT HL60 cells (a). The translocation peak was delayed in P2 compared to P1 and WT HL60 cells. Fig 15 is representative example of two independent experiments of WT HL60 cells, two P1 HL60 cell lines (P1a, P1b) and two P2 HL60 cell lines (P2a, P2b).

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 (Fig.17).

h. HL60 TRAIL-resistant clones are similar to P1 but not P2 HL60 cell lines

To exclude possible effect of TRAIL on HL60 cell differentiation and maturation during the selection of the TRAIL-resistant HL60 cell lines, we established TRAIL-resistant HL60 clones (n=5) that had never been exposed to TRAIL (see Materials and Methods). All HL60 clones showed suppressed sensitivity to TRAIL-induced apoptosis (Fig.18). Thus, in contrast to acquired TRAIL-resistance of P1 and P2 HL60 cell lines, TRAIL-resistance of HL60 clones was constitutive.

Figure 18

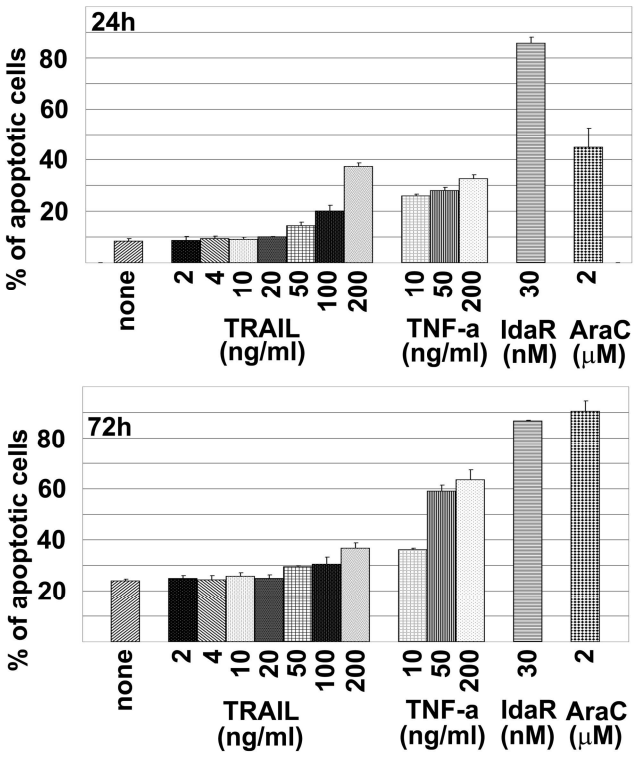


Fig.18 TRAIL-resistant HL60 clones show suppressed sensitivity to TRAIL, but remain sensitive to idarubicin (IdaR) and cytarabine (AraC). The level of apoptosis was measured by flow cytometry using FITC-conjugated Annexin-V assay 24, and 72 hours after addition of different concentrations of the cytotoxic agents (TRAIL, TNFα, idarubicin (IdaR) and cytarabine (AraC)). Columns represent means, and error bars standard deviations of values of three independent experiments.

Flow cytometry analysis of TRAIL-resistant HL60 clones showed moderate downregulation of all receptors for TRAIL, and low expression of CD14 (Fig.19).

Figure 19

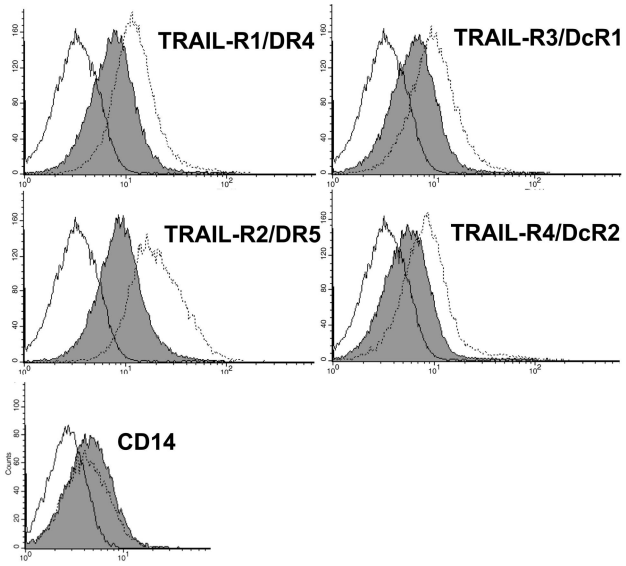


Fig.19 All receptors for TRAIL are downregulated and the expression of CD14 does not change in TRAIL-resistant HL60 clones compared to WT HL60 cells. Full histograms represent stain of the TRAIL-resistant HL60 clone. Interrupted open histograms represent stain of the WT HL60 cells. Continuous open histograms represent stain of the TRAIL-resistant HL60 clones with secondary antibody only. Data presented are representative example of a phenotype of five TRAIL-resistant HL60 clones.

On mRNA level, TRAIL-R2/DR5 and TRAIL-R3/DcR1 were downregulated (2-fold and 1.6-fold, respectively), while TRAIL-R1/DR4 and TRAIL-R4/DcR2 were upregulated (2-fold and 2.3-fold, respectively). There was significantly higher transcription of mRNA for TRAIL (21.3-fold) and osteoprotegerin (23.6-fold) in TRAIL-resistant HL60 clones compared to WT, P1 or P2 HL60 cells. FADD was prominently downregulated (944.4-fold) in TRAIL-resistant clones (Fig.20).

Figure 20

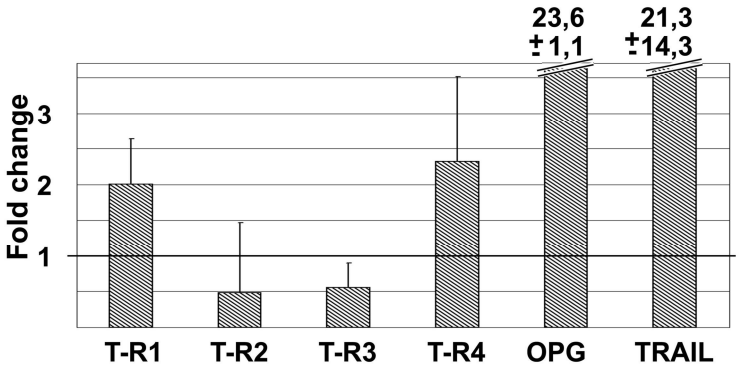


Fig.20 Real-time RT-PCR analysis of TRAIL and TRAIL-receptor mRNA expression of TRAIL-resistant HL60 clones. Real-time RT-PCR was carried out on WT HL60 cells and TRAIL-resistant clones. Figure 13 shows relative amounts of mRNA in TRAIL-resistant HL60 clones (dark columns) compared to relative amount of mRNA in WT HL60 cells (=1). WT HL60 cells, and two TRAIL-resistant HL60 clones were analyzed. Gene expressions of TRAIL, TRAIL-R1, 2, 3, 4, and OPG were analyzed. Columns represent means, and error bars standard deviations of values of four independent experiments (two experiments for each cell lines).

Real-time RT-PCR analysis unveiled marked gene expression pattern differences between TRAIL-resistant clones and WT HL60 cells. We detected significant (>2-fold) mRNA overexpression of antiapoptotic XIAP (288,3-fold), cIAP1 (8,9-fold), cIAP2 (7,4-fold), NAIP (4,7-fold), cFLIP-L (2,7-fold), BCL2 (2,3-fold), and MCL1 (2,2-fold), but also upregulation of proapoptotic BID (13,6-fold), BAD (5,7-fold), and CASP8 (4,2-fold) in TRAIL-resistant HL60 clones. mRNA levels of antiapoptotic genes survivin and BCL-XL were downregulated (3,1-fold and 2,1-fold, respectively) (Fig.21).

Figure 21

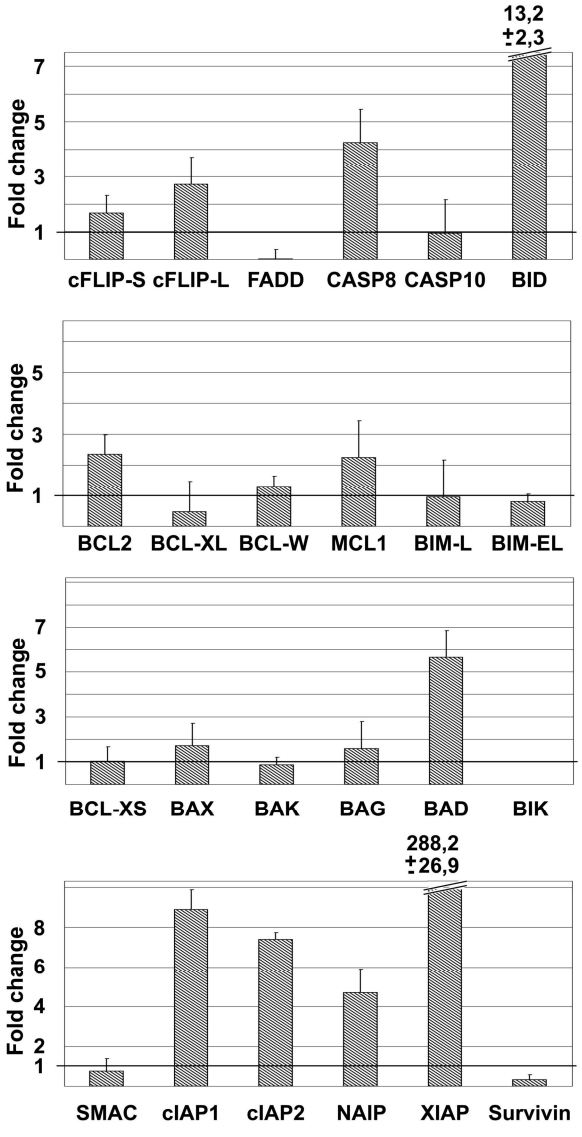


Fig.21 Expression pattern of important apoptosis regulators is altered in TRAIL-resistant HL60 clones compared to WT HL60 cells. Figure 19 shows relative amounts of mRNA in TRAIL-resistant clones compared to relative amount of mRNA in WT HL60 cells (=1). WT HL60 cells, and two TRAIL-resistant clones were analyzed. Gene expressions of the 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 clone).

TRAIL-induced translocation of RelA/p65 transcription factor to the nucleus of TRAIL-exposed TRAIL-resistant clones 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 constitutive TRAIL-resistance was not confirmed (Fig.22).

Figure 22

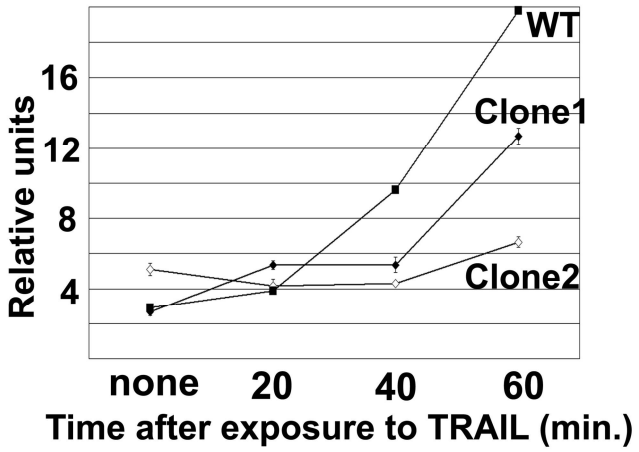


Fig.22 NFκB signaling is suppressed in TRAIL-resistant HL60 clones compared to WT HL60 cells. Cells were lysed 20, 40, and 60 minutes after exposure to TRAIL (200ng/mL), and nuclear translocations of NFκB member RelA/p65 was analyzed by ELISA-based NFκB translocation assay. We observed significant decrease of the NFκB translocation peaks in TRAIL-resistant HL60 clones (Clone1, 2) compared to WT HL60 cells (WT). Fig 20 is representative example of two independent experiments of WT HL60 cells and two TRAIL-resistant clones.

Our results suggest existence of a distinct population of TRAIL-resistant HL60 cells, as exemplified by TRAIL-resistant HL60 clones, in the original WT HL60 cell line. Analyses of TRAIL-resistant HL60 clones suggest important similarities with P1, but not with P2, phenotype of TRAIL-resistant HL60 cell lines.

i. 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 (Fig.23). WT HL60 injected mice did not develop either hind-leg paralysis or macroscopic intraperitoneal tumors (Tab.3). Xenotransplantation of P1 cells into the mice did not significantly differ from that of WT HL60 cells (Tab.3). 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 (Fig.24), 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; $p \leq 0.01$; Tab.3).

Table 3

	Sacrificed on Day	Mouse Weight	Hind-Leg Paralysis	Number of Peripheral Tumors	Number of Intra-Peritoneal Tumors	Tumor Weight	Tumor Infiltration	Bone Marrow Infiltration	Brain Infiltration	Liver Infiltration
WT HL60_#1	39,0	17,5	No	1	none	0,6	25%	15%	22%	none
WT HL60_#2	39,0	22,8	No	0	none	NA	NA	none	22%	none
WT HL60_#3	41,0	23,6	No	2	none	1,1	31%	5%	30%	2%
WT HL60_#4	42,0	25,4	No	0	none	NA	NA	6%	15%	none
WT HL60_#5	42,0	22,4	No	0	none	NA	NA	3%	27%	none
WT HL60_#6	45,0	19,2	No	1	none	0,8	19%	none	35%	3%
MEAN	**41,3**	**21,8**			**none**	**0,8**	**25%**	**7%**	**25%**	**2%**
P1 HL60_#1	38,0	21,1	No	none	none	NA	24%	5%	42%	none
P1 HL60_#2	41,0	18,5	No	none	none	NA	21%	none	26%	none
P1 HL60_#3	41,0	17,7	No	1	none	0,4	19%	11%	28%	3%
P1 HL60_#4	40,0	16,9	No	1	none	0,6	12%	9%	36%	3%
P1 HL60_#5	40,0	16,0	No	2	none	0,3	18%	19%	53%	4%
P1 HL60_#6	43,0	20,4	No	1	none	0,3	20%	none	46%	none
MEAN	**40,5**	**18,4**			**none**	**0,4**	**19%**	**11%**	**39%**	**3%**
P2 HL60_#1	33,0	20,8	Yes	none	3,0	4,1	64%	4%	15%	3%
P2 HL60_#2	33,0	21,0	Yes	1	2,0	3,3	55%	28%	12%	3%
P2 HL60_#3	36,0	22,4	Yes	none	2,0	3,1	82%	15%	17%	2%
P2 HL60_#4	27,0	17,1	No	none	2,0	1,2	70%	1%	35%	1%
P2 HL60_#5	27,0	18,7	No	none	2,0	1,4	82%	2%	22%	none
P2 HL60_#6	29,0	17,2	No	1	2,0	2,6	66%	2%	31%	none
MEAN	**30,8**	**19,5**			**2,2**	**2,6**	**70%**	**9%**	**22%**	**2%**

Tab.3 Xenotransplantation of P2 HL60 cells into immunodeficient NOD/LtSz-Rag1^{null} mice is associated with shortened survival compared to P1 or WT HL60 cells. Legend in text and in Materials and Methods.

Figure 23

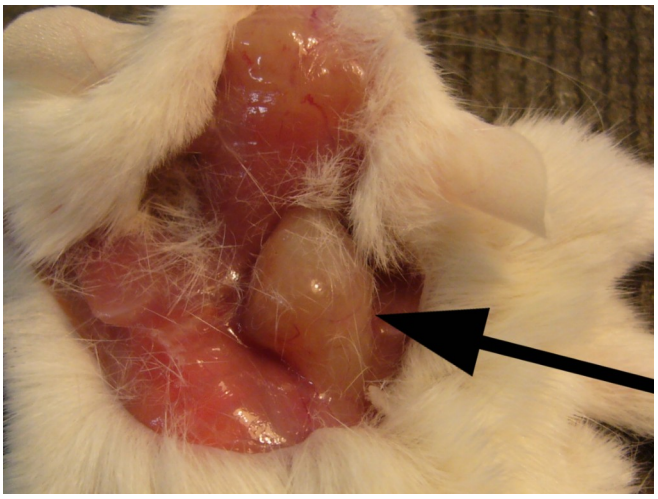


Fig.23 Peripheral lymph node enlargement of an immunodeficient mouse xenotransplanted with P1 HL60 cells.

Figure 24

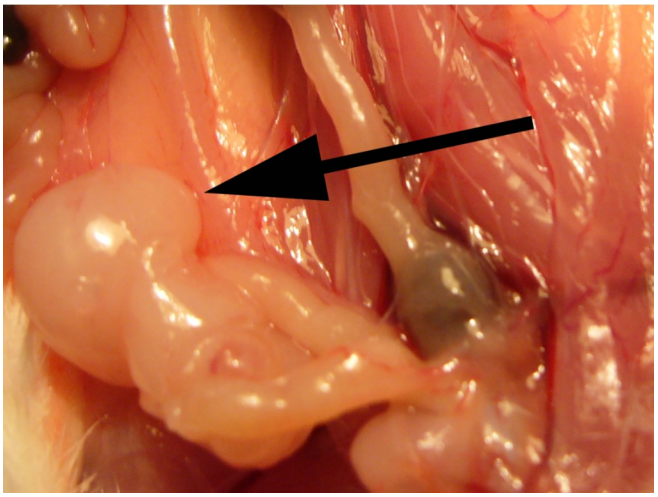


Fig.24 Intraperitoneal tumor of an immunodeficient mouse xenotransplanted with P2 HL60 cells.

HL60 cell lines re-established from HL60 leukemia xenotransplanted animals were analyzed. Xenotransplanted WT HL60 cells retained low CD14 expression and sensitivity to TRAIL. Xenotransplanted P2 HL60 cells retained CD14 positivity and complete TRAIL resistance. Xenotransplanted P1 HL60 cells retained low CD14 antigen, but sensitivity to TRAIL was partially restored (44±15% apoptosis 24 hours after the exposure to TRAIL 200ng/mL). The same observations were obtained with long-term *in vitro* cultures of P1 and P2 HL60 cells (data not shown). Thus, TRAIL resistance of P1 HL60 cells, unlike the TRAIL resistance of P2 HL60 cells, was not stable in time.

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

To analyze potential cytotoxic synergy of TRAIL and cytostatics currently used for the treatment of acute myeloid leukemia, cytarabine (AraC) and idarubicin (IdaR), we measured the effects of diverse therapeutic strategies comprising different combinations and/or concentrations of the TRAIL, cytarabine and idarubicin, on WT HL60 cell proliferation and survival (Fig.25).

Figure 25

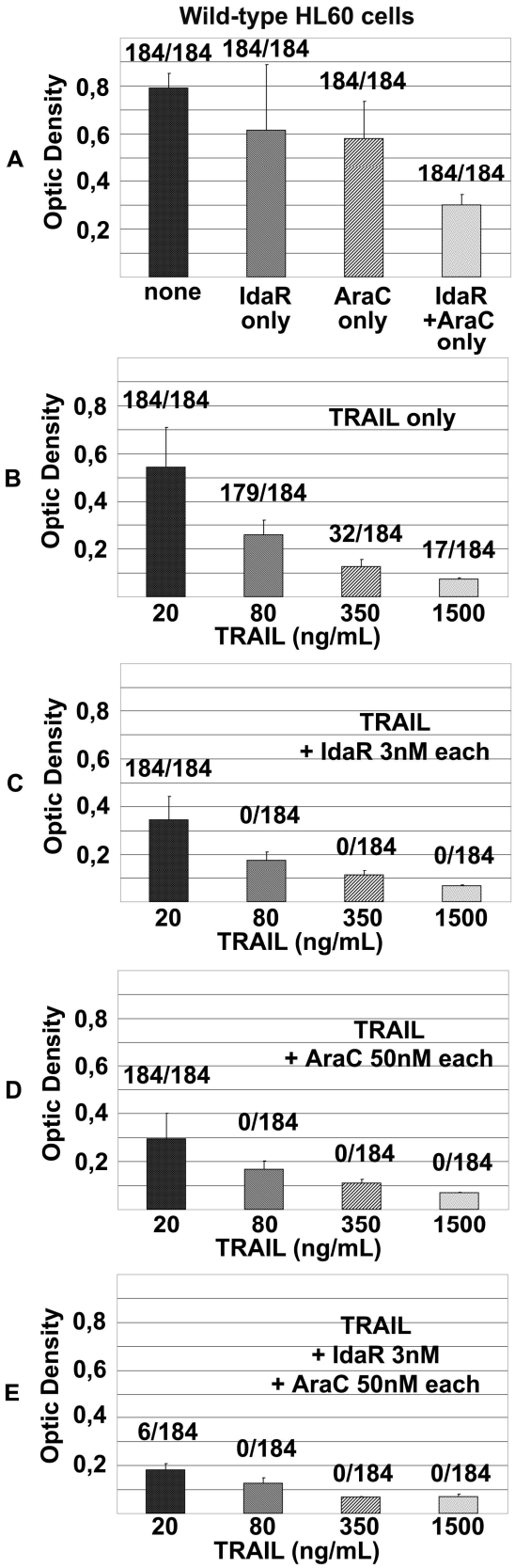


Fig.25 TRAIL potentiates the cytotoxic effects of cytarabine and idarubicin on WT HL60 leukemia cells. For each concentration/combination of the drugs, a column represents mean ± standard deviation of values of MTT proliferation assays of twelve wells on day 8. Fraction number above each column represents proportion of wells containing living cells on day 30 (e.g. 32/184 means that from 184 wells examined on day 30, 32 wells contained proliferating cells, and 152 wells dead cells). Fig.19 shows that in contrast to single-agent TRAIL, idarubicin, or cytarabine, combining TRAIL with idarubicin and/or cytarabine not only more potently suppresses leukemia cell proliferation, but most importantly leads to eradication of "leukemia units".

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

4. 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. Alternatively, TRAIL-resistant HL60 cell clones were derived without exposure of the cells to TRAIL.

In various cancer cell lines and primary tumor cells TRAIL exerted programmed cell death either via death receptor TRAIL-R1/DR4, death receptor TRAIL-R2/DR5 or the both death receptors [309-313]. Our experiments showed that the death receptor TRAIL-R1/DR4 transduces TRAIL death signal more potently than the death receptor TRAIL-R2/DR5 in WT HL60 cells.

Decoy receptors (TRAIL-R3/DcR1, TRAIL-R4/DcR2) or osteoprotegerin were implicated in constitutive or acquired resistance to TRAIL [247-250,314]. In the present study, however, we did not find evidence for the involvement of decoy receptors (TRAIL-R3/DcR1, TRAIL-R4/DcR2, or OPG) in TRAIL resistance of HL60 cells.

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. We identified distinct molecular and biological differences between 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 [315]. 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 several mechanisms of TRAIL resistance development. Either WT HL60 cells did not undergo apoptosis because of alterations in TRAIL-induced apoptotic pathway, including defects in the expression of TRAIL-receptors (P1 cells), or TRAIL triggered non-apoptotic signaling pathways that resulted in the change of the cell phenotype (P2 cells). The former mechanism would imply presence of TRAIL resistant cells in the WT HL60 cell line. To test this hypothesis we derived TRAIL-resistant HL60 clones from individual WT HL60 cells. The TRAIL-resistant HL60 clones had low CD14 and detectable expression of OPG, similar to WT and P2 HL60 cells. Similarly to P1 cells, TRAIL resistant HL60 clones showed significant upregulation of several members of antiapoptotic IAP and BCL-2 gene families. The findings thus supported the hypothesis that P1 cells were selected as a result of TRAIL-mediated elimination of TRAIL-sensitive WT HL60 cells.

Recently, acquired resistance to TRAIL in human ovarian carcinoma cell line was associated with resistance to Fas-ligand (FasL), but not to chemotherapeutic agents [316]. In several other studies, acquired TRAIL-resistance of malignant cells was associated with alterations of receptor mediated apoptosis, namely mutations of caspase 8, increased turnover of caspase 8, increased expression of cFLIP or disruption of FADD [140,172,254-260]. 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. Similarly to TRAIL, 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 and compared the ability of TRAIL and TNFα to induce caspase 8 and 10 activation in TRAIL resistant and WT HL60 cells. In TRAIL-sensitive 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 activity of caspase 8 and 10 after the challenge with TRAIL or TNFα, which is in concordance with decreased expression of TRAIL and TNFα receptors. While in P2 HL60 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 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 [146,317-319]. 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. Thus, it can be reasoned that treating leukemia with single-agent TRAIL might be associated with a high risk of relapse/progression

of the disease brought about by TRAIL-resistant leukemia cell clones that might possibly exert higher biologic aggressivity, as exemplified on TRAIL-resistant P2 HL60 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 [83,83,86,90,198-220]. 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.

The data from this study suggest that targeting of both, intrinsic and extrinsic, apoptotic pathways may be essential for the development of novel combinatorial strategies in the treatment of leukemia and other malignancies.

5. CONCLUSIONS

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

II. 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.**

III. 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.

IV. 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.

V. 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

VI. 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**,.

VII. 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.

VIII. 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.

IX. 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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7. REFERENCES

(1) Fink SL, Cookson BT. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. Infect Immun. 2005;73:1907-1916.

(2) Golstein P, Kroemer G. Cell death by necrosis: towards a molecular definition. Trends Biochem Sci. 2007;32:37-43.

(3) Fadeel B, Orrenius S. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. J Intern Med. 2005;258:479-517.

(4) Rossi D, Gaidano G. Messengers of cell death: apoptotic signaling in health and disease. Haematologica. 2003;88:212-218.

(5) Petak I, Houghton JA. Shared pathways: death receptors and cytotoxic drugs in cancer therapy. Pathol Oncol Res. 2001;7:95-106.

(6) Thorburn A. Death receptor-induced cell killing. Cell Signal. 2004;16:139-144.

(7) Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science. 2004;305:626-629.

(8) Ferrari D, Stepczynska A, Los M, Wesselborg S, Schulze-Osthoff K. Differential regulation and ATP requirement for caspase-8 and caspase-3 activation during. J Exp Med. 1998;188:979-984.

(9) Galluzzi L, Larochette N, Zamzami N, Kroemer G. Mitochondria as therapeutic targets for cancer chemotherapy. Oncogene. 2006;25:4812-4830.

(10) Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. Physiol Rev. 2007;87:99-163.

(11) Yang J, Liu X, Bhalla K et al. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science. 1997;275:1129-1132.

(12) Harris MH, Thompson CB. The role of the Bcl-2 family in the regulation of outer mitochondrial membrane permeability. Cell Death Differ. 2000;7:1182-1191.

(13) Saelens X, Festjens N, Vande WL et al. Toxic proteins released from mitochondria in cell death. Oncogene. 2004;23:2861-2874.

(14) Garrido C, Galluzzi L, Brunet M et al. Mechanisms of cytochrome c release from mitochondria. Cell Death Differ. 2006;13:1423-1433.

(15) Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell. 1996;86:147-157.

(16) Lorenzo HK, Susin SA, Penninger J, Kroemer G. Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death. Cell Death Differ. 1999;6:516-524.

(17) Susin SA, Lorenzo HK, Zamzami N et al. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature. 1999;397:441-446.

(18) Arnoult D, Gaume B, Karbowski M et al. Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. EMBO J. 2003;22:4385-4399.

(19) Shi Y. Apoptosome: the cellular engine for the activation of caspase-9. Structure. 2002;10:285-288.

(20) Chinnaiyan AM. The apoptosome: heart and soul of the cell death machine. Neoplasia. 1999;1:5-15.

(21) Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell. 1997;90:405-413.

(22) Porter AG, Janicke RU. Emerging roles of caspase-3 in apoptosis. Cell Death Differ. 1999;6:99-104.

(23) Slee EA, Adrain C, Martin SJ. Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. J Biol Chem. 2001;276:7320-7326.

(24) Tucker SJ, Rae C, Littlejohn AF, Paul A, MacEwan DJ. Switching leukemia cell phenotype between life and death. Proc Natl Acad Sci U S A. 2004;101:12940-12945.

(25) MacEwan DJ. TNF ligands and receptors--a matter of life and death. Br J Pharmacol. 2002;135:855-875.

(26) Ashkenazi A, Dixit VM. Apoptosis control by death and decoy receptors. Curr Opin Cell Biol. 1999;11:255-260.

(27) Pan G, O'Rourke K, Chinnaiyan AM et al. The receptor for the cytotoxic ligand TRAIL. Science. 1997;276:111-113.

(28) Wajant H. Death receptors. Essays Biochem. 2003;39:53-71.

(29) Walczak H, Degli-Esposti MA, Johnson RS et al. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J. 1997;16:5386-5397.

(30) Sheridan JP, Marsters SA, Pitti RM et al. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science. 1997;277:818-821.

(31) Shipman CM, Croucher PI. Osteoprotegerin is a soluble decoy receptor for tumor necrosis factor-related apoptosis-inducing ligand/Apo2 ligand and can function as a paracrine survival factor for human myeloma cells. Cancer Res. 2003;63:912-916.

(32) Emery JG, McDonnell P, Burke MB et al. Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. J Biol Chem. 1998;273:14363-14367.

(33) Pan G, Ni J, Wei YF et al. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science. 1997;277:815-818.

(34) Pan G, Ni J, Yu G, Wei YF, Dixit VM. TRUNDD, a new member of the TRAIL receptor family that antagonizes TRAIL signalling. FEBS Lett. 1998;424:41-45.

(35) Pitti RM, Marsters SA, Ruppert S et al. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J Biol Chem. 1996;271:12687-12690.

(36) Hymowitz SG, O'Connell MP, Ultsch MH et al. A unique zinc-binding site revealed by a high-resolution X-ray structure of homotrimeric Apo2L/TRAIL. Biochemistry. 2000;39:633-640.

(37) Seol DW, Li J, Seol MH et al. Signaling events triggered by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL): caspase-8 is required for TRAIL-induced apoptosis. Cancer Res. 2001;61:1138-1143.

(38) Kischkel FC, Lawrence DA, Chuntharapai A et al. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. Immunity. 2000;12:611-620.

(39) Kischkel FC, Lawrence DA, Tinel A et al. Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. J Biol Chem. 2001;276:46639-46646.

(40) Sakahira H, Enari M, Nagata S. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature. 1998;391:96-99.

(41) Enari M, Sakahira H, Yokoyama H et al. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature. 1998;391:43-50.

(42) Nagata S. Apoptotic DNA fragmentation. Exp Cell Res. 2000;256:12-18.

(43) Nagata S, Nagase H, Kawane K, Mukae N, Fukuyama H. Degradation of chromosomal DNA during apoptosis. Cell Death Differ. 2003;10:108-116.

(44) Boulares AH, Yakovlev AG, Ivanova V et al. Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. J Biol Chem. 1999;274:22932-22940.

(45) Nunez G, Benedict MA, Hu Y, Inohara N. Caspases: the proteases of the apoptotic pathway. Oncogene. 1998;17:3237-3245.

(46) Slee EA, Adrain C, Martin SJ. Serial killers: ordering caspase activation events in apoptosis. Cell Death Differ. 1999;6:1067-1074.

(47) Boatright KM, Renatus M, Scott FL et al. A unified model for apical caspase activation. Mol Cell. 2003;11:529-541.

(48) Pop C, Timmer J, Sperandio S, Salvesen GS. The apoptosome activates caspase-9 by dimerization. Mol Cell. 2006;22:269-275.

(49) Salvesen GS, Dixit VM. Caspase activation: the induced-proximity model. Proc Natl Acad Sci U S A. 1999;96:10964-10967.

(50) Chang DW, Ditsworth D, Liu H et al. Oligomerization is a general mechanism for the activation of apoptosis initiator and inflammatory procaspases. J Biol Chem. 2003;278:16466-16469.

(51) Sahara S, Aoto M, Eguchi Y et al. Acinus is a caspase-3-activated protein required for apoptotic chromatin condensation. Nature. 1999;401:168-173.

(52) Kivinen K, Kallajoki M, Taimen P. Caspase-3 is required in the apoptotic disintegration of the nuclear matrix. Exp Cell Res. 2005;311:62-73.

(53) Janicke RU, Ng P, Sprengart ML, Porter AG. Caspase-3 is required for alpha-fodrin cleavage but dispensable for cleavage of other death substrates in apoptosis. J Biol Chem. 1998;273:15540-15545.

(54) Rao L, Perez D, White E. Lamin proteolysis facilitates nuclear events during apoptosis. J Cell Biol. 1996;135:1441-1455.

(55) Slee EA, Adrain C, Martin SJ. Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. J Biol Chem. 2001;276:7320-7326.

(56) Kihlmark M, Imreh G, Hallberg E. Sequential degradation of proteins from the nuclear envelope during apoptosis. J Cell Sci. 2001;114:3643-3653.

(57) Kass GE, Eriksson JE, Weis M, Orrenius S, Chow SC. Chromatin condensation during apoptosis requires ATP. Biochem J. 1996;318 (Pt 3):749-752.

(58) Eguchi Y, Srinivasan A, Tomaselli KJ, Shimizu S, Tsujimoto Y. ATP-dependent steps in apoptotic signal transduction. Cancer Res. 1999;59:2174-2181.

(59) Simbulan-Rosenthal CM, Rosenthal DS, Iyer S, Boulares AH, Smulson ME. Transient poly(ADP-ribosyl)ation of nuclear proteins and role of poly(ADP-ribose) polymerase in the early stages of apoptosis. J Biol Chem. 1998;273:13703-13712.

(60) Eguchi Y, Shimizu S, Tsujimoto Y. Intracellular ATP levels determine cell death fate by apoptosis or necrosis. Cancer Res. 1997;57:1835-1840.

(61) Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell. 1998;94:481-490.

(62) Yin XM, Wang K, Gross A et al. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature. 1999;400:886-891.

(63) Ruffolo SC, Breckenridge DG, Nguyen M et al. BID-dependent and BID-independent pathways for BAX insertion into mitochondria. Cell Death Differ. 2000;7:1101-1108.

(64) Ozoren N, El Deiry WS. Defining characteristics of Types I and II apoptotic cells in response to TRAIL. Neoplasia. 2002;4:551-557.

(65) Yin XM. Signal transduction mediated by Bid, a pro-death Bcl-2 family proteins, connects the death receptor and mitochondria apoptosis pathways. Cell Res. 2000;10:161-167.

(66) Ozoren N, El Deiry WS. Defining characteristics of Types I and II apoptotic cells in response to TRAIL. Neoplasia. 2002;4:551-557.

(67) Ozoren N, Kim K, Burns TF et al. The caspase 9 inhibitor Z-LEHD-FMK protects human liver cells while permitting death of cancer cells exposed to tumor necrosis factor-related apoptosis-inducing ligand. Cancer Res. 2000;60:6259-6265.

(68) Varfolomeev E, Maecker H, Sharp D et al. Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand. J Biol Chem. 2005;280:40599-40608.

(69) Lin Y, Devin A, Cook A et al. The death domain kinase RIP is essential for TRAIL (Apo2L)-induced activation of IkappaB kinase and c-Jun N-terminal kinase. Mol Cell Biol. 2000;20:6638-6645.

(70) Kim YS, Schwabe RF, Qian T, Lemasters JJ, Brenner DA. TRAIL-mediated apoptosis requires NF-kappaB inhibition and the mitochondrial permeability transition in human hepatoma cells. Hepatology. 2002;36:1498-1508.

(71) Muhlenbeck F, Haas E, Schwenzer R et al. TRAIL/Apo2L activates c-Jun NH2-terminal kinase (JNK) via caspase-dependent and caspase-independent pathways. J Biol Chem. 1998;273:33091-33098.

(72) Liu J, Lin A. Role of JNK activation in apoptosis: a double-edged sword. Cell Res. 2005;15:36-42.

(73) Papa S, Zazzeroni F, Pham CG, Bubici C, Franzoso G. Linking JNK signaling to NF-kappaB: a key to survival. J Cell Sci. 2004;117:5197-5208.

(74) Bubici C, Papa S, Pham CG, Zazzeroni F, Franzoso G. NF-kappaB and JNK: an intricate affair. Cell Cycle. 2004;3:1524-1529.

(75) Papa S, Bubici C, Zazzeroni F et al. The NF-kappaB-mediated control of the JNK cascade in the antagonism of programmed cell death in health and disease. Cell Death Differ. 2006;13:712-729.

(76) Liu J, Lin A. Role of JNK activation in apoptosis: a double-edged sword. Cell Res. 2005;15:36-42.

(77) Heyninck K, Beyaert R. Crosstalk between NF-kappaB-activating and apoptosis-inducing proteins of the TNF-receptor complex. Mol Cell Biol Res Commun. 2001;4:259-265.

(78) Jeremias I, Debatin KM. TRAIL induces apoptosis and activation of NFkappaB. Eur Cytokine Netw. 1998;9:687-688.

(79) Haupt S, Berger M, Goldberg Z, Haupt Y. Apoptosis - the p53 network. J Cell Sci. 2003;116:4077-4085.

(80) Sheikh MS, Burns TF, Huang Y et al. p53-dependent and -independent regulation of the death receptor KILLER/DR5 gene expression in response to genotoxic stress and tumor necrosis factor alpha. Cancer Res. 1998;58:1593-1598.

(81) Wu GS, Burns TF, Zhan Y, Alnemri ES, El Deiry WS. Molecular cloning and functional analysis of the mouse homologue of the KILLER/DR5 tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor. Cancer Res. 1999;59:2770-2775.

(82) Zou W, Liu X, Yue P et al. c-Jun NH2-terminal kinase-mediated up-regulation of death receptor 5 contributes to induction of apoptosis by the novel synthetic triterpenoid methyl-2-cyano-3,12-dioxooleana-1, 9-dien-28-oate in human lung cancer cells. Cancer Res. 2004;64:7570-7578.

(83) Kondo K, Yamasaki S, Sugie T et al. Cisplatin-dependent upregulation of death receptors 4 and 5 augments induction of apoptosis by TNF-related apoptosis-inducing ligand against esophageal squamous cell carcinoma. Int J Cancer. 2006;118:230-242.

(84) Broaddus VC, Dansen TB, Abayasiriwardana KS et al. Bid mediates apoptotic synergy between tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and DNA damage. J Biol Chem. 2005;280:12486-12493.

(85) Jazirehi AR, Ng CP, Gan XH, Schiller G, Bonavida B. Adriamycin sensitizes the adriamycin-resistant 8226/Dox40 human multiple myeloma cells to Apo2L/tumor necrosis factor-related apoptosis-inducing ligand-mediated (TRAIL) apoptosis. Clin Cancer Res. 2001;7:3874-3883.

(86) Keane MM, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. Cancer Res. 1999;59:734-741.

(87) Basu A, Castle VP, Bouziane M, Bhalla K, Haldar S. Crosstalk between extrinsic and intrinsic cell death pathways in pancreatic cancer: synergistic action of estrogen metabolite and ligands of death receptor family. Cancer Res. 2006;66:4309-4318.

(88) Marini P, Schmid A, Jendrossek V et al. Irradiation specifically sensitises solid tumour cell lines to TRAIL mediated apoptosis. BMC Cancer. 2005;5:5.

(89) Ma Y, Lakshmikanthan V, Lewis RW, Kumar MV. Sensitization of TRAIL-resistant cells by inhibition of heat shock protein 90 with low-dose geldanamycin. Mol Cancer Ther. 2006;5:170-178.

(90) Broaddus VC, Dansen TB, Abayasiriwardana KS et al. Bid mediates apoptotic synergy between tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and DNA damage. J Biol Chem. 2005;280:12486-12493.

(91) Galligan L, Longley DB, McEwan M et al. Chemotherapy and TRAIL-mediated colon cancer cell death: the roles of p53, TRAIL receptors, and c-FLIP. Mol Cancer Ther. 2005;4:2026-2036.

(92) Brooks AD, Sayers TJ. Reduction of the antiapoptotic protein cFLIP enhances the susceptibility of human renal cancer cells to TRAIL apoptosis. Cancer Immunol Immunother. 2005;54:499-505.

(93) Siegmund D, Hadwiger P, Pfizenmaier K, Vornlocher HP, Wajant H. Selective inhibition of FLICE-like inhibitory protein expression with small interfering RNA oligonucleotides is sufficient to sensitize tumor cells for TRAIL-induced apoptosis. Mol Med. 2002;8:725-732.

(94) Okano H, Shiraki K, Inoue H et al. Cellular FLICE/caspase-8-inhibitory protein as a principal regulator of cell death and survival in human hepatocellular carcinoma. Lab Invest. 2003;83:1033-1043.

(95) Zhang X, Jin TG, Yang H et al. Persistent c-FLIP(L) expression is necessary and sufficient to maintain resistance to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in prostate cancer. Cancer Res. 2004;64:7086-7091.

(96) Mathas S, Lietz A, Anagnostopoulos I et al. c-FLIP mediates resistance of Hodgkin/Reed-Sternberg cells to death receptor-induced apoptosis. J Exp Med. 2004;199:1041-1052.

(97) Zhang S, Shen HM, Ong CN. Down-regulation of c-FLIP contributes to the sensitization effect of 3,3'-diindolylmethane on TRAIL-induced apoptosis in cancer cells. Mol Cancer Ther. 2005;4:1972-1981.

(98) Ricci MS, Jin Z, Dews M et al. Direct repression of FLIP expression by c-myc is a major determinant of TRAIL sensitivity. Mol Cell Biol. 2004;24:8541-8555.

(99) Letai A. Pharmacological manipulation of Bcl-2 family members to control cell death. J Clin Invest. 2005;115:2648-2655.

(100) Green DR. At the gates of death. Cancer Cell. 2006;9:328-330.

(101) Reed JC. Bcl-2 family proteins. Oncogene. 1998;17:3225-3236.

(102) Heiser D, Labi V, Erlacher M, Villunger A. The Bcl-2 protein family and its role in the development of neoplastic disease. Exp Gerontol. 2004;39:1125-1135.

(103) Letai A, Sorcinelli MD, Beard C, Korsmeyer SJ. Antiapoptotic BCL-2 is required for maintenance of a model leukemia. Cancer Cell. 2004;6:241-249.

(104) Beere HM. "The stress of dying": the role of heat shock proteins in the regulation of apoptosis. J Cell Sci. 2004;117:2641-2651.

(105) Beere HM. Death versus survival: functional interaction between the apoptotic and stress-inducible heat shock protein pathways. J Clin Invest. 2005;115:2633-2639.

(106) Utz PJ, Anderson P. Life and death decisions: regulation of apoptosis by proteolysis of signaling molecules. Cell Death Differ. 2000;7:589-602.

(107) Kregel KC. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. J Appl Physiol. 2002;92:2177-2186.

(108) Gabai VL, Mabuchi K, Mosser DD, Sherman MY. Hsp72 and stress kinase c-jun N-terminal kinase regulate the bid-dependent pathway in tumor necrosis factor-induced apoptosis. Mol Cell Biol. 2002;22:3415-3424.

(109) Ran R, Lu A, Zhang L et al. Hsp70 promotes TNF-mediated apoptosis by binding IKK gamma and impairing NF-kappa B survival signaling. Genes Dev. 2004;18:1466-1481.

(110) Garrido C, Schmitt E, Cande C et al. HSP27 and HSP70: potentially oncogenic apoptosis inhibitors. Cell Cycle. 2003;2:579-584.

(111) Shiozaki EN, Chai J, Rigotti DJ et al. Mechanism of XIAP-mediated inhibition of caspase-9. Mol Cell. 2003;11:519-527.

(112) Srinivasula SM, Hegde R, Saleh A et al. A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. Nature. 2001;410:112-116.

(113) Deveraux QL, Reed JC. IAP family proteins--suppressors of apoptosis. Genes Dev. 1999;13:239-252.

(114) Verhagen AM, Coulson EJ, Vaux DL. Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. Genome Biol. 2001;2:REVIEWS3009.

(115) de Graaf AO, de Witte T, Jansen JH. Inhibitor of apoptosis proteins: new therapeutic targets in hematological cancer? Leukemia. 2004;18:1751-1759.

(116) Schimmer AD. Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. Cancer Res. 2004;64:7183-7190.

(117) Srinivasula SM, Datta P, Fan XJ et al. Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway. J Biol Chem. 2000;275:36152-36157.

(118) Verhagen AM, Ekert PG, Pakusch M et al. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell. 2000;102:43-53.

(119) Almond JB, Cohen GM. The proteasome: a novel target for cancer chemotherapy. Leukemia. 2002;16:433-443.

(120) Ciechanover A. The ubiquitin-proteasome pathway: on protein death and cell life. EMBO J. 1998;17:7151-7160.

(121) Orlowski RZ. The role of the ubiquitin-proteasome pathway in apoptosis. Cell Death Differ. 1999;6:303-313.

(122) Voorhees PM, Dees EC, O'Neil B, Orlowski RZ. The proteasome as a target for cancer therapy. Clin Cancer Res. 2003;9:6316-6325.

(123) Buchsbaum DJ, Zhou T, Lobuglio AF. TRAIL receptor-targeted therapy. Future Oncol. 2006;2:493-508.

(124) Reed JC. Drug insight: cancer therapy strategies based on restoration of endogenous cell death mechanisms. Nat Clin Pract Oncol. 2006;3:388-398.

(125) Rowinsky EK. Targeted induction of apoptosis in cancer management: the emerging role of tumor necrosis factor-related apoptosis-inducing ligand receptor activating agents. J Clin Oncol. 2005;23:9394-9407.

(126) Fischer U, Schulze-Osthoff K. Apoptosis-based therapies and drug targets. Cell Death Differ. 2005;12 Suppl 1:942-961.

(127) Wu XX, Ogawa O, Kakehi Y. TRAIL and chemotherapeutic drugs in cancer therapy. Vitam Horm. 2004;67:365-383.

(128) Gruss HJ. Molecular, structural, and biological characteristics of the tumor necrosis factor ligand superfamily. Int J Clin Lab Res. 1996;26:143-159.

(129) Wiley SR, Schooley K, Smolak PJ et al. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity. 1995;3:673-682.

(130) Hymowitz SG, Christinger HW, Fuh G et al. Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5. Mol Cell. 1999;4:563-571.

(131) Takeda K, Hayakawa Y, Smyth MJ et al. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat Med. 2001;7:94-100.

(132) Takeda K, Smyth MJ, Cretney E et al. Critical role for tumor necrosis factor-related apoptosis-inducing ligand in immune surveillance against tumor development. J Exp Med. 2002;195:161-169.

(133) Zerafa N, Westwood JA, Cretney E et al. Cutting edge: TRAIL deficiency accelerates hematological malignancies. J Immunol. 2005;175:5586-5590.

(134) Ashkenazi A, Pai RC, Fong S et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest. 1999;104:155-162.

(135) Wajant H, Pfizenmaier K, Scheurich P. TNF-related apoptosis inducing ligand (TRAIL) and its receptors in tumor surveillance and cancer therapy. Apoptosis. 2002;7:449-459.

(136) Spierings DC, de Vries EG, Vellenga E et al. Tissue distribution of the death ligand TRAIL and its receptors. J Histochem Cytochem. 2004;52:821-831.

(137) Daniels RA, Turley H, Kimberley FC et al. Expression of TRAIL and TRAIL receptors in normal and malignant tissues. Cell Res. 2005;15:430-438.

(138) Gura T. How TRAIL kills cancer cells, but not normal cells. Science. 1997;277:768.

(139) Kruidering M, Evan GI. Caspase-8 in apoptosis: the beginning of "the end"? IUBMB Life. 2000;50:85-90.

(140) Knight MJ, Riffkin CD, Ekert PG, Ashley DM, Hawkins CJ. Caspase-8 levels affect necessity for mitochondrial amplification in death ligand-induced glioma cell apoptosis. Mol Carcinog. 2004;39:173-182.

(141) Engels IH, Totzke G, Fischer U, Schulze-Osthoff K, Janicke RU. Caspase-10 sensitizes breast carcinoma cells to TRAIL-induced but not tumor necrosis factor-induced apoptosis in a caspase-3-dependent manner. Mol Cell Biol. 2005;25:2808-2818.

(142) Sprick MR, Rieser E, Stahl H et al. Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. EMBO J. 2002;21:4520-4530.

(143) Shin S, Lee Y, Kim W et al. Caspase-2 primes cancer cells for TRAIL-mediated apoptosis by processing procaspase-8. EMBO J. 2005;24:3532-3542.

(144) Kluttermann K, Banning U, Kachel M et al. TRAIL-induced cytotoxicity in a melphalan-resistant rhabdomyosarcoma cell line via activation of caspase-2. Anticancer Res. 2006;26:351-356.

(145) Kemp TJ, Kim JS, Crist SA, Griffith TS. Induction of necrotic tumor cell death by TRAIL/Apo-2L. Apoptosis. 2003;8:587-599.

(146) Malhi H, Gores GJ. TRAIL resistance results in cancer progression: a TRAIL to perdition? Oncogene. 2006;25:7333-7335.

(147) Meurette O, Huc L, Rebillard A et al. TRAIL (TNF-related apoptosis-inducing ligand) induces necrosis-like cell death in tumor cells at acidic extracellular pH. Ann N Y Acad Sci. 2005;1056:379-387.

(148) Walczak H, Miller RE, Ariail K et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med. 1999;5:157-163.

(149) Hao C, Song JH, Hsi B et al. TRAIL inhibits tumor growth but is nontoxic to human hepatocytes in chimeric mice. Cancer Res. 2004;64:8502-8506.

(150) Kelley SK, Harris LA, Xie D et al. Preclinical studies to predict the disposition of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand in humans: characterization of in vivo efficacy, pharmacokinetics, and safety. J Pharmacol Exp Ther. 2001;299:31-38.

(151) Ganten TM, Koschny R, Sykora J et al. Preclinical differentiation between apparently safe and potentially hepatotoxic applications of TRAIL either alone or in combination with chemotherapeutic drugs. Clin Cancer Res. 2006;12:2640-2646.

(152) Koschny R, Ganten TM, Sykora J et al. TRAIL/bortezomib cotreatment is potentially hepatotoxic but induces cancer-specific apoptosis within a therapeutic window. Hepatology. 2007;45:649-658.

(153) Abdollahi T. Potential for TRAIL as a therapeutic agent in ovarian cancer. Vitam Horm. 2004;67:347-364.

(154) Ahmad M, Shi Y. TRAIL-induced apoptosis of thyroid cancer cells: potential for therapeutic intervention. Oncogene. 2000;19:3363-3371.

(155) Bradbury J. TRAIL leads to apoptosis in acute promyelocytic leukaemia. Lancet. 2001;357:1770.

(156) Cillessen SA, Meijer CJ, Ossenkoppele GJ et al. Human soluble TRAIL/Apo2L induces apoptosis in a subpopulation of chemotherapy refractory nodal diffuse large B-cell lymphomas, determined by a highly sensitive in vitro apoptosis assay. Br J Haematol. 2006;134:283-293.

(157) Zauli G, Sancilio S, Cataldi A et al. PI-3K/Akt and NF-kappaB/IkappaBalpha pathways are activated in Jurkat T cells in response to TRAIL treatment. J Cell Physiol. 2005;202:900-911.

(158) Plasilova M, Zivny J, Jelinek J et al. TRAIL (Apo2L) suppresses growth of primary human leukemia and myelodysplasia progenitors. Leukemia. 2002;16:67-73.

(159) Hylander BL, Pitoniak R, Penetrante RB et al. The anti-tumor effect of Apo2L/TRAIL on patient pancreatic adenocarcinomas grown as xenografts in SCID mice. J Transl Med. 2005;3:22.

(160) Jalving M, de Jong S, Koornstra JJ et al. TRAIL induces apoptosis in human colorectal adenoma cell lines and human colorectal adenomas. Clin Cancer Res. 2006;12:4350-4356.

(161) Kagawa S, He C, Gu J et al. Antitumor activity and bystander effects of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene. Cancer Res. 2001;61:3330-3338.

(162) LeBlanc HN, Ashkenazi A. Apo2L/TRAIL and its death and decoy receptors. Cell Death Differ. 2003;10:66-75.

(163) Kimberley FC, Screaton GR. Following a TRAIL: update on a ligand and its five receptors. Cell Res. 2004;14:359-372.

(164) Chaudhary PM, Eby M, Jasmin A et al. Death receptor 5, a new member of the TNFR family, and DR4 induce FADD-dependent apoptosis and activate the NF-kappaB pathway. Immunity. 1997;7:821-830.

(165) Degli-Esposti MA, Dougall WC, Smolak PJ et al. The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. Immunity. 1997;7:813-820.

(166) Degli-Esposti MA, Smolak PJ, Walczak H et al. Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. J Exp Med. 1997;186:1165-1170.

(167) Kuijlen JM, Mooij JJ, Platteel I et al. TRAIL-receptor expression is an independent prognostic factor for survival in patients with a primary glioblastoma multiforme. J Neurooncol. 2006;78:161-171.

(168) Strater J, Hinz U, Walczak H et al. Expression of TRAIL and TRAIL receptors in colon carcinoma: TRAIL-R1 is an independent prognostic parameter. Clin Cancer Res. 2002;8:3734-3740.

(169) McCarthy MM, Sznol M, DiVito KA et al. Evaluating the expression and prognostic value of TRAIL-R1 and TRAIL-R2 in breast cancer. Clin Cancer Res. 2005;11:5188-5194.

(170) McCarthy MM, DiVito KA, Sznol M et al. Expression of tumor necrosis factor--related apoptosis-inducing ligand receptors 1 and 2 in melanoma. Clin Cancer Res. 2006;12:3856-3863.

(171) Bodmer JL, Holler N, Reynard S et al. TRAIL receptor-2 signals apoptosis through FADD and caspase-8. Nat Cell Biol. 2000;2:241-243.

(172) Kuang AA, Diehl GE, Zhang J, Winoto A. FADD is required for DR4- and DR5-mediated apoptosis: lack of trail-induced apoptosis in FADD-deficient mouse embryonic fibroblasts. J Biol Chem. 2000;275:25065-25068.

(173) Walczak H, Krammer PH. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. Exp Cell Res. 2000;256:58-66.

(174) Muhlenbeck F, Haas E, Schwenzer R et al. TRAIL/Apo2L activates c-Jun NH2-terminal kinase (JNK) via caspase-dependent and caspase-independent pathways. J Biol Chem. 1998;273:33091-33098.

(175) Muhlenbeck F, Schneider P, Bodmer JL et al. The tumor necrosis factor-related apoptosis-inducing ligand receptors TRAIL-R1 and TRAIL-R2 have distinct cross-linking requirements for initiation of apoptosis and are non-redundant in JNK activation. J Biol Chem. 2000;275:32208-32213.

(176) Schneider P, Thome M, Burns K et al. TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-kappaB. Immunity. 1997;7:831-836.

(177) Abdollahi T, Robertson NM, Abdollahi A, Litwack G. Inhibition of TRAIL-induced apoptosis by IL-8 is mediated by the p38-MAPK pathway in OVCAR3 cells. Apoptosis. 2005;10:1383-1393.

(178) Varfolomeev E, Maecker H, Sharp D et al. Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand. J Biol Chem. 2005;280:40599-40608.

(179) Lin Y, Devin A, Cook A et al. The death domain kinase RIP is essential for TRAIL (Apo2L)-induced activation of IkappaB kinase and c-Jun N-terminal kinase. Mol Cell Biol. 2000;20:6638-6645.

(180) Varfolomeev EE, Ashkenazi A. Tumor necrosis factor: an apoptosis JuNKie? Cell. 2004;116:491-497.

(181) Shaulian E, Karin M. AP-1 in cell proliferation and survival. Oncogene. 2001;20:2390-2400.

(182) Mathas S, Hinz M, Anagnostopoulos I et al. Aberrantly expressed c-Jun and JunB are a hallmark of Hodgkin lymphoma cells, stimulate proliferation and synergize with NF-kappa B. EMBO J. 2002;21:4104-4113.

(183) Fujioka S, Niu J, Schmidt C et al. NF-kappaB and AP-1 connection: mechanism of NF-kappaB-dependent regulation of AP-1 activity. Mol Cell Biol. 2004;24:7806-7819.

(184) Lin Y, Devin A, Rodriguez Y, Liu ZG. Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. Genes Dev. 1999;13:2514-2526.

(185) Lin Y, Devin A, Cook A et al. The death domain kinase RIP is essential for TRAIL (Apo2L)-induced activation of IkappaB kinase and c-Jun N-terminal kinase. Mol Cell Biol. 2000;20:6638-6645.

(186) Lee NK, Lee SY. Modulation of life and death by the tumor necrosis factor receptor-associated factors (TRAFs). J Biochem Mol Biol. 2002;35:61-66.

(187) Lee SY, Reichlin A, Santana A et al. TRAF2 is essential for JNK but not NF-kappaB activation and regulates lymphocyte proliferation and survival. Immunity. 1997;7:703-713.

(188) Jin Z, El Deiry WS. Distinct signaling pathways in T. Mol Cell Biol. 2006;26:8136-8148.

(189) Marsters SA, Sheridan JP, Pitti RM et al. A novel receptor for Apo2L/TRAIL contains a truncated death domain. Curr Biol. 1997;7:1003-1006.

(190) Merino D, Lalaoui N, Morizot A et al. Differential inhibition of TRAIL-mediated DR5-DISC formation by decoy receptors 1 and 2. Mol Cell Biol. 2006;26:7046-7055.

(191) Lee HW, Lee SH, Lee HW et al. Homomeric and heteromeric interactions of the extracellular domains of death receptors and death decoy receptors. Biochem Biophys Res Commun. 2005;330:1205-1212.

(192) Chan FK, Chun HJ, Zheng L et al. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. Science. 2000;288:2351-2354.

(193) Clancy L, Mruk K, Archer K et al. Preligand assembly domain-mediated ligand-independent association between TRAIL receptor 4 (TR4) and TR2 regulates TRAIL-induced apoptosis. Proc Natl Acad Sci U S A. 2005;102:18099-18104.

(194) Krieg A, Krieg T, Wenzel M et al. TRAIL-beta and TRAIL-gamma: two novel splice variants of the human TNF-related apoptosis-inducing ligand (TRAIL) without apoptotic potential. Br J Cancer. 2003;88:918-927.

(195) Debatin KM, Poncet D, Kroemer G. Chemotherapy: targeting the mitochondrial cell death pathway. Oncogene. 2002;21:8786-8803.

(196) Sheikh MS, Fornace AJ, Jr. Death and decoy receptors and p53-mediated apoptosis. Leukemia. 2000;14:1509-1513.

(197) Walczak H, Bouchon A, Stahl H, Krammer PH. Tumor necrosis factor-related apoptosis-inducing ligand retains its apoptosis-inducing capacity on Bcl-2- or Bcl-xL-overexpressing chemotherapy-resistant tumor cells. Cancer Res. 2000;60:3051-3057.

(198) Macfarlane M, Cohen GM, Dickens M. JNK (c-Jun N-terminal kinase) and p38 activation in receptor-mediated and chemically-induced apoptosis of T-cells: differential requirements for caspase activation. Biochem J. 2000;348 Pt 1:93-101.

(199) Marini P, Schmid A, Jendrossek V et al. Irradiation specifically sensitises solid tumour cell lines to TRAIL mediated apoptosis. BMC Cancer. 2005;5:5.

(200) Keane MM, Rubinstein Y, Cuello M et al. Inhibition of NF-kappaB activity enhances TRAIL mediated apoptosis in breast cancer cell lines. Breast Cancer Res Treat. 2000;64:211-219.

(201) Kim EH, Kim SU, Shin DY, Choi KS. Roscovitine sensitizes glioma cells to TRAIL-mediated apoptosis by downregulation of survivin and XIAP. Oncogene. 2004;23:446-456.

(202) Kim EH, Kim HS, Kim SU et al. Sodium butyrate sensitizes human glioma cells to TRAIL-mediated apoptosis through inhibition of Cdc2 and the subsequent downregulation of survivin and XIAP. Oncogene. 2005;24:6877-6889.

(203) Kim EH, Kim SU, Choi KS. Rottlerin sensitizes glioma cells to TRAIL-induced apoptosis by inhibition of Cdc2 and the subsequent downregulation of survivin and XIAP. Oncogene. 2005;24:838-849.

(204) Kleeff J, Kornmann M, Sawhney H, Korc M. Actinomycin D induces apoptosis and inhibits growth of pancreatic cancer cells. Int J Cancer. 2000;86:399-407.

(205) Akay C, Gazitt Y. Arsenic trioxide selectively induces early and extensive apoptosis via the APO2/caspase-8 pathway engaging the mitochondrial pathway in myeloma cells with mutant p53. Cell Cycle. 2003;2:358-368.

(206) Brooks AD, Ramirez T, Toh U et al. The proteasome inhibitor bortezomib (Velcade) sensitizes some human tumor cells to Apo2L/TRAIL-mediated apoptosis. Ann N Y Acad Sci. 2005;1059:160-167.

(207) Chinnaiyan AM, Prasad U, Shankar S et al. Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. Proc Natl Acad Sci U S A. 2000;97:1754-1759.

(208) Cuello M, Ettenberg SA, Nau MM, Lipkowitz S. Synergistic induction of apoptosis by the combination of trail and chemotherapy in chemoresistant ovarian cancer cells. Gynecol Oncol. 2001;81:380-390.

(209) El Zawahry A, McKillop J, Voelkel-Johnson C. Doxorubicin increases the effectiveness of Apo2L/TRAIL for tumor growth inhibition of prostate cancer xenografts. BMC Cancer. 2005;5:2.

(210) Inoue S, Macfarlane M, Harper N et al. Histone deacetylase inhibitors potentiate TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in lymphoid malignancies. Cell Death Differ. 2004;11 Suppl 2:S193-S206.

(211) Inoue T, Shiraki K, Fuke H et al. Proteasome inhibition sensitizes hepatocellular carcinoma cells to TRAIL by suppressing caspase inhibitors and AKT pathway. Anticancer Drugs. 2006;17:261-268.

(212) Keane MM, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. Cancer Res. 1999;59:734-741.

(213) Matsuzaki H, Schmied BM, Ulrich A et al. Combination of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and actinomycin D induces apoptosis even in TRAIL-resistant human pancreatic cancer cells. Clin Cancer Res. 2001;7:407-414.

(214) Nakata S, Yoshida T, Horinaka M et al. Histone deacetylase inhibitors upregulate death receptor 5/TRAIL-R2 and sensitize apoptosis induced by TRAIL/APO2-L in human malignant tumor cells. Oncogene. 2004;23:6261-6271.

(215) Nikrad M, Johnson T, Puthalalath H et al. The proteasome inhibitor bortezomib sensitizes cells to killing by death receptor ligand TRAIL via BH3-only proteins Bik and Bim. Mol Cancer Ther. 2005;4:443-449.

(216) Nimmanapalli R, Porosnicu M, Nguyen D et al. Cotreatment with STI-571 enhances tumor necrosis factor alpha-related apoptosis-inducing ligand (TRAIL or apo-2L)-induced apoptosis of Bcr-Abl-positive human acute leukemia cells. Clin Cancer Res. 2001;7:350-357.

(217) Nimmanapalli R, Perkins CL, Orlando M et al. Pretreatment with paclitaxel enhances apo-2 ligand/tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis of prostate cancer cells by inducing death receptors 4 and 5 protein levels. Cancer Res. 2001;61:759-763.

(218) Odoux C, Albers A. Additive effects of TRAIL and paclitaxel on cancer cells: implications for advances in cancer therapy. Vitam Horm. 2004;67:385-407.

(219) Wendt J, von Haefen C, Hemmati P et al. TRAIL sensitizes for ionizing irradiation-induced apoptosis through an entirely Bax-dependent mitochondrial cell death pathway. Oncogene. 2005;24:4052-4064.

(220) Ballestrero A, Nencioni A, Boy D et al. Tumor necrosis factor-related apoptosis-inducing ligand cooperates with anticancer drugs to overcome chemoresistance in antiapoptotic Bcl-2 family members expressing jurkat cells. Clin Cancer Res. 2004;10:1463-1470.

(221) Ng CP, Zisman A, Bonavida B. Synergy is achieved by complementation with Apo2L/TRAIL and actinomycin D in Apo2L/TRAIL-mediated apoptosis of prostate cancer cells: role of XIAP in resistance. Prostate. 2002;53:286-299.

(222) Marini P, Belka C. Death receptor ligands: new strategies for combined treatment with ionizing radiation. Curr Med Chem Anticancer Agents. 2003;3:334-342.

(223) Kim DM, Koo SY, Jeon K et al. Rapid induction of apoptosis by combination of flavopiridol and tumor necrosis factor (TNF)-alpha or TNF-related apoptosis-inducing ligand in human cancer cell lines. Cancer Res. 2003;63:621-626.

(224) Lee NS, Cheong HJ, Kim SJ et al. Ex vivo purging of leukemia cells using tumor-necrosis-factor-related apoptosis-inducing ligand in hematopoietic stem cell transplantation. Leukemia. 2003;17:1375-1383.

(225) Pukac L, Kanakaraj P, Humphreys R et al. HGS-ETR1, a fully human TRAIL-receptor 1 monoclonal antibody, induces cell death in multiple tumour types in vitro and in vivo. Br J Cancer. 2005;92:1430-1441.

(226) Georgakis GV, Li Y, Humphreys R et al. Activity of selective fully human agonistic antibodies to the TRAIL death receptors TRAIL-R1 and TRAIL-R2 in primary and cultured lymphoma cells: induction of apoptosis and enhancement of doxorubicin- and bortezomib-induced cell death. Br J Haematol. 2005;130:501-510.

(227) Motoki K, Mori E, Matsumoto A et al. Enhanced apoptosis and tumor regression induced by a direct agonist antibody to tumor necrosis factor-related apoptosis-inducing ligand receptor 2. Clin Cancer Res. 2005;11:3126-3135.

(228) Kazhdan I, Marciniak RA. Death receptor 4 (DR4) efficiently kills breast cancer cells irrespective of their sensitivity to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Cancer Gene Ther. 2004;11:691-698.

(229) Gajewski TF. On the TRAIL toward death receptor-based cancer therapeutics. J Clin Oncol. 2007;25:1305-1307.

(230) Liu Z, Xu X, Hsu HC et al. CII-DC-AdTRAIL cell gene therapy inhibits infiltration of CII-reactive T cells and CII-induced arthritis. J Clin Invest. 2003;112:1332-1341.

(231) Kim CY, Jeong M, Mushiake H et al. Cancer gene therapy using a novel secretable trimeric TRAIL. Gene Ther. 2006;13:330-338.

(232) El Zawahry A, Lu P, White SJ, Voelkel-Johnson C. In vitro efficacy of AdTRAIL gene therapy of bladder cancer is enhanced by trichostatin A-mediated restoration of CAR expression and downregulation of cFLIP and Bcl-XL. Cancer Gene Ther. 2006;13:281-289.

(233) Song K, Benhaga N, Anderson RL, Khosravi-Far R. Transduction of tumor necrosis factor-related apoptosis-inducing ligand into hematopoietic cells leads to inhibition of syngeneic tumor growth in vivo. Cancer Res. 2006;66:6304-6311.

(234) Aydin C, Sanlioglu AD, Karacay B et al. Decoy receptor-2 small interfering RNA (siRNA) strategy employing three different siRNA constructs in combination defeats adenovirus-transferred tumor necrosis factor-related apoptosis-inducing ligand resistance in lung cancer cells. Hum Gene Ther. 2007;18:39-50.

(235) Wenger T, Mattern J, Haas TL et al. Apoptosis mediated by lentiviral TRAIL transfer involves transduction-dependent and -independent effects. Cancer Gene Ther. 2007;14:316-326.

(236) Matsubara H, Mizutani Y, Hongo F et al. Gene therapy with TRAIL against renal cell carcinoma. Mol Cancer Ther. 2006;5:2165-2171.

(237) Yang F, Shi P, Xi X et al. Recombinant adenoviruses expressing TRAIL demonstrate antitumor effects on non-small cell lung cancer (NSCLC). Med Oncol. 2006;23:191-204.

(238) Ishii M, Iwai M, Harada Y et al. Soluble TRAIL gene and actinomycin D synergistically suppressed multiple metastasis of TRAIL-resistant colon cancer in the liver. Cancer Lett. 2007;245:134-143.

(239) Wenger T, Mattern J, Penzel R et al. Specific resistance upon lentiviral TRAIL transfer by intracellular retention of TRAIL receptors. Cell Death Differ. 2006;13:1740-1751.

(240) Vanoosten RL, Earel JK, Jr., Griffith TS. Enhancement of Ad5-TRAIL cytotoxicity against renal cell carcinoma with histone deacetylase inhibitors. Cancer Gene Ther. 2006;13:628-632.

(241) Ma H, Liu Y, Liu S, Xu R, Zheng D. Oral adeno-associated virus-sTRAIL gene therapy suppresses human hepatocellular carcinoma growth in mice. Hepatology. 2005;42:1355-1363.

(242) Yoo J, Choi S, Hwang KS et al. Adeno-associated virus-mediated gene transfer of a secreted form of TRAIL inhibits tumor growth and occurrence in an experimental tumor model. J Gene Med. 2006;8:163-174.

(243) Sato K, Nakaoka T, Yamashita N et al. TRAIL-transduced dendritic cells protect mice from acute graft-versus-host disease and leukemia relapse. J Immunol. 2005;174:4025-4033.

(244) Lee SH, Shin MS, Kim HS et al. Somatic mutations of TRAIL-receptor 1 and TRAIL-receptor 2 genes in non-Hodgkin's lymphoma. Oncogene. 2001;20:399-403.

(245) Rubio-Moscardo F, Blesa D, Mestre C et al. Characterization of 8p21.3 chromosomal deletions in B-cell lymphoma: TRAIL-R1 and TRAIL-R2 as candidate dosage-dependent tumor suppressor genes. Blood. 2005;106:3214-3222.

(246) Liu LG, Tanaka H, Ito K et al. Absence of gene mutation in TRAIL receptor 1 (TRAIL-R1) and TRAIL receptor 2 (TRAIL-R2) in chronic myelogenous leukemia and myelodysplastic syndrome, and analysis of mRNA Expressions of TRAIL and TRAIL-related genes in chronic myelogenous leukemia. Acta Haematol. 2005;113:113-123.

(247) Bruserud O. TRAIL decoy receptors mediate resistance of acute myeloid leukemia cells to TRAIL. Haematologica. 2005;90:579.

(248) Riccioni R, Pasquini L, Mariani G et al. TRAIL decoy receptors mediate resistance of acute myeloid leukemia cells to TRAIL. Haematologica. 2005;90:612-624.

(249) Liao Q, Friess H, Kleeff J, Buchler MW. Differential expression of TRAIL-R3 and TRAIL-R4 in human pancreatic cancer. Anticancer Res. 2001;21:3153-3159.

(250) Fayad R, Brand MI, Stone D, Keshavarzian A, Qiao L. Apoptosis resistance in ulcerative colitis: high expression of decoy receptors by lamina propria T cells. Eur J Immunol. 2006;36:2215-2222.

(251) Holen I, Croucher PI, Hamdy FC, Eaton CL. Osteoprotegerin (OPG) is a survival factor for human prostate cancer cells. Cancer Res. 2002;62:1619-1623.

(252) Holen I, Cross SS, Neville-Webbe HL et al. Osteoprotegerin (OPG) expression by breast cancer cells in vitro and breast tumours in vivo--a role in tumour cell survival? Breast Cancer Res Treat. 2005;92:207-215.

(253) Neville-Webbe HL, Cross NA, Eaton CL et al. Osteoprotegerin (OPG) produced by bone marrow stromal cells protects breast cancer cells from TRAIL-induced apoptosis. Breast Cancer Res Treat. 2004;86:269-279.

(254) Zhang L, Zhu H, Teraishi F et al. Accelerated degradation of caspase-8 protein correlates with TRAIL resistance in a DLD1 human colon cancer cell line. Neoplasia. 2005;7:594-602.

(255) Eggert A, Grotzer MA, Zuzak TJ et al. Resistance to TRAIL-induced apoptosis in neuroblastoma cells correlates with a loss of caspase-8 expression. Med Pediatr Oncol. 2000;35:603-607.

(256) Ganten TM, Haas TL, Sykora J et al. Enhanced caspase-8 recruitment to and activation at the DISC is critical for sensitisation of human hepatocellular carcinoma cells to TRAIL-induced apoptosis by chemotherapeutic drugs. Cell Death Differ. 2004;11 Suppl 1:S86-S96.

(257) Garnett TO, Filippova M, Duerksen-Hughes PJ. Accelerated degradation of FADD and procaspase 8 in cells expressing human papilloma virus 16 E6 impairs TRAIL-mediated apoptosis. Cell Death Differ. 2006;13:1915-1926.

(258) Hopkins-Donaldson S, Bodmer JL, Bourloud KB et al. Loss of caspase-8 expression in neuroblastoma is related to malignancy and resistance to TRAIL-induced apoptosis. Med Pediatr Oncol. 2000;35:608-611.

(259) Kanehara I, Nakata B, Hirakawa K. Caspase-8 is scarcely silenced and its activity is well correlated with the anticancer effect of tumor necrosis factor-related apoptosis-inducing ligand in gastric cancer cells. Oncol Rep. 2005;14:1249-1253.

(260) Tamagiku Y, Sonoda Y, Kunisawa M et al. Down-regulation of procaspase-8 expression by focal adhesion kinase protects HL-60 cells from TRAIL-induced apoptosis. Biochem Biophys Res Commun. 2004;323:445-452.

(261) Roth W, Reed JC. FLIP protein and TRAIL-induced apoptosis. Vitam Horm. 2004;67:189-206.

(262) Guicciardi ME, Bronk SF, Werneburg NW, Gores GJ. c-FLIPL Prevents TRAIL-Induced Apoptosis of Hepatocellular Carcinoma Cells by Inhibiting the Lysosomal Pathway of Apoptosis. Am J Physiol Gastrointest Liver Physiol. 2007.

(263) Jonsson G, Paulie S, Grandien A. High level of cFLIP correlates with resistance to death receptor-induced apoptosis in bladder carcinoma cells. Anticancer Res. 2003;23:1213-1218.

(264) Kreuz S, Siegmund D, Scheurich P, Wajant H. NF-kappaB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. Mol Cell Biol. 2001;21:3964-3973.

(265) Mathas S, Lietz A, Anagnostopoulos I et al. c-FLIP mediates resistance of Hodgkin/Reed-Sternberg cells to death receptor-induced apoptosis. J Exp Med. 2004;199:1041-1052.

(266) Okano H, Shiraki K, Inoue H et al. Cellular FLICE/caspase-8-inhibitory protein as a principal regulator of cell death and survival in human hepatocellular carcinoma. Lab Invest. 2003;83:1033-1043.

(267) Chawla-Sarkar M, Bae SI, Reu FJ et al. Downregulation of Bcl-2, FLIP or IAPs (XIAP and survivin) by siRNAs sensitizes resistant melanoma cells to Apo2L/TRAIL-induced apoptosis. Cell Death Differ. 2004;11:915-923.

(268) Hietakangas V, Poukkula M, Heiskanen KM et al. Erythroid differentiation sensitizes K562 leukemia cells to TRAIL-induced apoptosis by downregulation of c-FLIP. Mol Cell Biol. 2003;23:1278-1291.

(269) Azuhata T, Scott D, Griffith TS, Miller M, Sandler AD. Survivin inhibits apoptosis induced by TRAIL, and the ratio between survivin and TRAIL receptors is predictive of recurrent disease in neuroblastoma. J Pediatr Surg. 2006;41:1431-1440.

(270) Nakao K, Hamasaki K, Ichikawa T et al. Survivin downregulation by siRNA sensitizes human hepatoma cells to TRAIL-induced apoptosis. Oncol Rep. 2006;16:389-392.

(271) Georgakis GV, Li Y, Rassidakis GZ et al. Inhibition of heat shock protein 90 function by 17-allylamino-17-demethoxy-geldanamycin in Hodgkin's lymphoma cells down-regulates Akt kinase, dephosphorylates extracellular signal-regulated kinase, and induces cell cycle arrest and cell death. Clin Cancer Res. 2006;12:584-590.

(272) Lamothe B, Aggarwal BB. Ectopic expression of Bcl-2 and Bcl-xL inhibits apoptosis induced by TNF-related apoptosis-inducing ligand (TRAIL) through suppression of caspases-8, 7, and 3 and BID cleavage in human acute myelogenous leukemia cell line HL-60. J Interferon Cytokine Res. 2002;22:269-279.

(273) Sinicrope FA, Penington RC, Tang XM. Tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis is inhibited by Bcl-2 but restored by the small molecule Bcl-2 inhibitor, HA 14-1, in human colon cancer cells. Clin Cancer Res. 2004;10:8284-8292.

(274) Taniai M, Grambihler A, Higuchi H et al. Mcl-1 mediates tumor necrosis factor-related apoptosis-inducing ligand resistance in human cholangiocarcinoma cells. Cancer Res. 2004;64:3517-3524.

(275) Braeuer SJ, Buneker C, Mohr A, Zwacka RM. Constitutively activated nuclear factor-kappaB, but not induced NF-kappaB, leads to TRAIL resistance by up-regulation of X-linked inhibitor of apoptosis protein in human cancer cells. Mol Cancer Res. 2006;4:715-728.

(276) Chawla-Sarkar M, Bauer JA, Lupica JA et al. Suppression of NF-kappa B survival signaling by nitrosylcobalamin sensitizes neoplasms to the anti-tumor effects of Apo2L/TRAIL. J Biol Chem. 2003;278:39461-39469.

(277) Chen X, Kandasamy K, Srivastava RK. Differential roles of RelA (p65) and c-Rel subunits of nuclear factor kappa B in tumor necrosis factor-related apoptosis-inducing ligand signaling. Cancer Res. 2003;63:1059-1066.

(278) Jeremias I, Debatin KM. TRAIL induces apoptosis and activation of NFkappaB. Eur Cytokine Netw. 1998;9:687-688.

(279) Khanbolooki S, Nawrocki ST, Arumugam T et al. Nuclear factor-kappaB maintains TRAIL resistance in human pancreatic cancer cells. Mol Cancer Ther. 2006;5:2251-2260.

(280) Li F, Meng L, Xing H et al. Essential role of c-Jun-NH2-terminal kinase on synergy induction of apoptosis by TRAIL plus ADM in ADM resistant MCF-7/ADM cells. Apoptosis. 2006;11:1239-1246.

(281) Herr I, Wilhelm D, Bohler T, Angel P, Debatin KM. JNK/SAPK activity is not sufficient for anticancer therapy-induced apoptosis involving CD95-L, TRAIL and TNF-alpha. Int J Cancer. 1999;80:417-424.

(282) Mikami T, Koyama T, Koyama T et al. C-jun N-terminal kinase activation is required for apoptotic cell death induced by TNF-related apoptosis-inducing ligand plus DNA-damaging agents in sarcoma cell lines. Anticancer Res. 2006;26:1153-1160.

(283) Lee MW, Park SC, Yang YG et al. The involvement of reactive oxygen species (ROS) and p38 mitogen-activated protein (MAP) kinase in TRAIL/Apo2L-induced apoptosis. FEBS Lett. 2002;512:313-318.

(284) Harper N, Hughes MA, Farrow SN, Cohen GM, Macfarlane M. Protein kinase C modulates tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by targeting the apical events of death receptor signaling. J Biol Chem. 2003;278:44338-44347.

(285) Sarker M, Ruiz-Ruiz C, Lopez-Rivas A. Activation of protein kinase C inhibits TRAIL-induced caspases activation, mitochondrial events and apoptosis in a human leukemic T cell line. Cell Death Differ. 2001;8:172-181.

(286) Thiefes A, Wolter S, Mushinski JF et al. Simultaneous blockade of NFkappaB, JNK, and p38 MAPK by a kinase-inactive mutant of the protein kinase TAK1 sensitizes cells to apoptosis and affects a distinct spectrum of tumor necrosis factor [corrected] target genes. J Biol Chem. 2005;280:27728-27741.

(287) Nesterov A, Nikrad M, Johnson T, Kraft AS. Oncogenic Ras sensitizes normal human cells to tumor necrosis factor-alpha-related apoptosis-inducing ligand-induced apoptosis. Cancer Res. 2004;64:3922-3927.

(288) Drosopoulos KG, Roberts ML, Cermak L et al. Transformation by oncogenic RAS sensitizes human colon cells to TRAIL-induced apoptosis by up-regulating death receptor 4 and death receptor 5 through a MEK-dependent pathway. J Biol Chem. 2005;280:22856-22867.

(289) Nieminen AI, Partanen JI, Hau A, Klefstrom J. c-Myc primed mitochondria determine cellular sensitivity to TRAIL-induced apoptosis. EMBO J. 2007;26:1055-1067.

(290) Zhang L, Fang B. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. Cancer Gene Ther. 2005;12:228-237.

(291) Cheng J, Hylander BL, Baer MR, Chen X, Repasky EA. Multiple mechanisms underlie resistance of leukemia cells to Apo2 Ligand/TRAIL. Mol Cancer Ther. 2006;5:1844-1853.

(292) Bortul R, Tazzari PL, Cappellini A et al. Constitutively active Akt1 protects HL60 leukemia cells from TRAIL-induced apoptosis through a mechanism involving NF-kappaB activation and cFLIP(L) up-regulation. Leukemia. 2003;17:379-389.

(293) Chen X, Thakkar H, Tyan F et al. Constitutively active Akt is an important regulator of TRAIL sensitivity in prostate cancer. Oncogene. 2001;20:6073-6083.

(294) Clohessy JG, Zhuang J, de Boer J, Gil-Gomez G, Brady HJ. Mcl-1 interacts with truncated Bid and inhibits its induction of cytochrome c release and its role in receptor-mediated apoptosis. J Biol Chem. 2006;281:5750-5759.

(295) Cummins JM, Kohli M, Rago C et al. X-linked inhibitor of apoptosis protein (XIAP) is a nonredundant modulator of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in human cancer cells. Cancer Res. 2004;64:3006-3008.

(296) Dudognon C, Pendino F, Hillion J et al. Death receptor signaling regulatory function for telomerase: hTERT abolishes TRAIL-induced apoptosis, independently of telomere maintenance. Oncogene. 2004;23:7469-7474.

(297) Fandy TE, Ross DD, Gore SD, Srivastava RK. Flavopiridol synergizes TRAIL cytotoxicity by downregulation of FLIP(L). Cancer Chemother Pharmacol. 2006.

(298) Hernandez A, Thomas R, Smith F et al. Butyrate sensitizes human colon cancer cells to TRAIL-mediated apoptosis. Surgery. 2001;130:265-272.

(299) Kim YH, Lee YJ. TRAIL apoptosis is enhanced by quercetin through Akt dephosphorylation. J Cell Biochem. 2007;100:998-1009.

(300) Tran SE, Holmstrom TH, Ahonen M, Kahari VM, Eriksson JE. MAPK/ERK overrides the apoptotic signaling from Fas, TNF, and TRAIL receptors. J Biol Chem. 2001;276:16484-16490.

(301) Zhang XD, Borrow JM, Zhang XY, Nguyen T, Hersey P. Activation of ERK1/2 protects melanoma cells from TRAIL-induced apoptosis by inhibiting Smac/DIABLO release from mitochondria. Oncogene. 2003;22:2869-2881.

(302) O'Gorman DM, McKenna SL, McGahon AJ, Cotter TG. Inhibition of PI3-kinase sensitises HL60 human leukaemia cells to both chemotherapeutic drug- and Fas-induced apoptosis by a JNK independent pathway. Leuk Res. 2001;25:801-811.

(303) Ravi R, Bedi A. NF-kappaB in cancer--a friend turned foe. Drug Resist Updat. 2004;7:53-67.

(304) Turco MC, Romano MF, Petrella A et al. NF-kappaB/Rel-mediated regulation of apoptosis in hematologic malignancies and normal hematopoietic progenitors. Leukemia. 2004;18:11-17.

(305) Piva R, Belardo G, Santoro MG. NF-kappaB: a stress-regulated switch for cell survival. Antioxid Redox Signal. 2006;8:478-486.

(306) Karin M. Nuclear factor-kappaB in cancer development and progression. Nature. 2006;441:431-436.

(307) Dutta J, Fan Y, Gupta N, Fan G, Gelinas C. Current insights into the regulation of programmed cell death by NF-kappaB. Oncogene. 2006;25:6800-6816.

(308) Luo JL, Kamata H, Karin M. IKK/NF-kappaB signaling: balancing life and death--a new approach to cancer therapy. J Clin Invest. 2005;115:2625-2632.

(309) Hesry V, Piquet-Pellorce C, Travert M et al. Sensitivity of prostate cells to TRAIL-induced apoptosis increases with tumor progression: DR5 and caspase 8 are key players. Prostate. 2006;66:987-995.

(310) Mitsiades N, Poulaki V, Mitsiades C, Tsokos M. Ewing's sarcoma family tumors are sensitive to tumor necrosis factor-related apoptosis-inducing ligand and express death receptor 4 and death receptor 5. Cancer Res. 2001;61:2704-2712.

(311) Macfarlane M, Inoue S, Kohlhaas SL et al. Chronic lymphocytic leukemic cells exhibit apoptotic signaling via TRAIL-R1. Cell Death Differ. 2005;12:773-782.

(312) Kelley RF, Totpal K, Lindstrom SH et al. Receptor-selective mutants of apoptosis-inducing ligand 2/tumor necrosis factor-related apoptosis-inducing ligand reveal a greater contribution of death receptor (DR) 5 than DR4 to apoptosis signaling. J Biol Chem. 2005;280:2205-2212.

(313) Kurbanov BM, Geilen CC, Fecker LF, Orfanos CE, Eberle J. Efficient TRAIL-R1/DR4-mediated apoptosis in melanoma cells by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). J Invest Dermatol. 2005;125:1010-1019.

(314) Bouralexis S, Findlay DM, Atkins GJ et al. Progressive resistance of BTK-143 osteosarcoma cells to Apo2L/TRAIL-induced apoptosis is mediated by acquisition of DcR2/TRAIL-R4 expression: resensitisation with chemotherapy. Br J Cancer. 2003;89:206-214.

(315) Secchiero P, Gonelli A, Mirandola P et al. Tumor necrosis factor-related apoptosis-inducing ligand induces monocytic maturation of leukemic and normal myeloid precursors through a caspase-dependent pathway. Blood. 2002;100:2421-2429.

(316) Lane D, Cote M, Grondin R, Couture MC, Piche A. Acquired resistance to TRAIL-induced apoptosis in human ovarian cancer cells is conferred by increased turnover of mature caspase-3. Mol Cancer Ther. 2006;5:509-521.

(317) Baader E, Toloczko A, Fuchs U et al. Tumor necrosis factor-related apoptosis-inducing ligand-mediated proliferation of tumor cells with receptor-proximal apoptosis defects. Cancer Res. 2005;65:7888-7895.

(318) Ehrhardt H, Fulda S, Schmid I et al. TRAIL induced survival and proliferation in cancer cells resistant towards TRAIL-induced apoptosis mediated by NF-kappaB. Oncogene. 2003;22:3842-3852.

(319) Trauzold A, Siegmund D, Schniewind B et al. TRAIL promotes metastasis of human pancreatic ductal adenocarcinoma. Oncogene. 2006;25:7434-7439.

8. APPENDIX

Makram Merimi, Pavel Klener, Maud Szynal, Yvette Cleuter, Claude Bagnis, Pierre Kerkhofs, Arsene Burny, Philippe Martiat, Anne Van den Broeke: *Complete suppression of viral gene expression is associated with the onset and progression of lymphoid malignancy: observations in Bovine Leukemia Virus-infected sheep;* **Retrovirology** 2007, 4:51 (accepted 23Jul2007) (IF=4,3)

Makram Merimi, Pavel Klener, Maud Szynal, Yvette Cleuter, Gaëlle Wymeersch, Pierre Kerkhofs, Arsène Burny, Philippe Martiat and Anne Van den Broeke: *Suppression of viral gene expression in Bovine Leukemia Virus-1 associated B-cell malignancy: interplay of epigenetic modifications leading to chromatin with a repressive histone code;* **J Virol**, June 2007, p. 5929–5939 (IF=5,1)

P. Klener jr., L. Anděra, P. Klener, E. Nečas, J. Živný: *Cell Death Signalling Pathways in the Pathogenesis and Therapy of Haematologic Malignancies: Overview of Therapeutic Approaches*, **Folia Biol-Prague** 52 (2006), 119-136 (IF=0,72)

P. Klener jr., L. Anděra, P. Klener, E. Nečas, J. Živný: *Cell Death Signalling Pathways in the Pathogenesis and Therapy of Haematologic Malignancies: Overview of Apoptotic Pathways*, **Folia Biol-Prague** 52 (2006), 34-44 (IF=0,72)

Pavel Klener, Maud Szynal, Yvette Cleuter, Makram Merimi, Hugues Duvillier, Françoise Lallemand, Claude Bagnis, Philip Griebel, Christos Sotiriou, Arsène Burny, Philippe Martiat, and Anne Van den Broeke*: Insights into Gene Expression Changes Impacting B-Cell*

Transformation: Cross-Species Microarray Analysis of Bovine Leukemia Virus Tax-Responsive Genes in Ovine B Cells; **J Virol** 2006 Feb; 80(4): 1922-1938 (IF=5,39)

Fuchs O, Simakova O, Klener P, Cmejlova J, Zivny J, Jelinek J, Zavadil J, Stopka T: *Inhibition of Smad5 in Human Hematopoietic Progenitors Blocks Erythroid Differentiation Induced by BMP4*; **Blood Cells Mol Dis.** 2002 Mar-Apr;28(2):221-33 (IF=1,77)

Chini EN, Klener P Jn, Beers KW, Chini CC, Grande JP, Dousa TP: *Cyclic ADP-ribose metabolism in rat kidney: high capacity for synthesis in glomeruli*; **Kidney Int.** 1997 May; 51(5):1500-6 (IF=4)