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BACHELOR THESIS

Characterization and regulation of TRAIL-induced apoptosis of tumor cells

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Contents

1. Abstract

Last fifty years of the research in life sciences are marked with an immense progress in our understanding of molecular mechanisms and cellular signaling pathways, some of them hopefully exploitable in our struggle to improve treatment of most threatening diseases as cancer, stroke or neurodegeneration. In this sense one of the current biggest challenges is designing novel anti-tumor therapies that will eliminate cancer cells without having serious side-effects. To such novel therapies could also belong specific induction of tumor cell death/apoptosis by the Tumor necrosis factor-Related Apoptosis Inducing Ligand (TRAIL), recently characterized member of the TNF-α family. This thesis documents our current knowledge about TRAIL, induction and regulation of its signaling pathways as well as about its significance in an organism. As summarized in its the first part, induction and regulation of apoptosis is a multi-step process that requires fine tuning of a number of signaling pathways with a pivotal role of mitochondria. Triggering of extrinsic or intrinsic apoptotic signaling leads to activation of proteases (as caspases), nucleases and finally to ordered destruction of the affected cell. The second part of the thesis is focused on in detail characterization of TRAIL-induced apoptotic signaling, its regulation and on analysis of acquired TRAIL resistance of tumor cells (including cancer stem cells) as a serious obstacle to effective, TRAIL-assisted tumor therapy.

Abstrakt

Obrovský pokrok v biomedicínckých oborech příznačný pro posledních padesát let zahrnuje poznání četných molekulárních mechanizmů, z nichž některé snad budou využitelné v boji proti nemocem jako rakovina, mrtvice či neurodegenerativní syndromy. Velkou výzvu v tomto směru představuje vývoj nových protinádorových terapií s minimálními vedlejšími účinky. Jednou takovou terapií by se mohlo stát selektivní navození programované buněčné smrti nádorových buněk pomocí molekuly TRAIL (česky: apoptózu vyvolávající ligand příbuzný s faktorem nekrotizujícím nádory). Tato práce podává přehled současných znalostí o TRAILu, spouštění a regulaci jeho signálních drah a jejich významu v organizmu. Jak je popsáno v první části této práce apoptóza představuje vícekrokový proces, který vyžaduje přesné vyladění a četné signální dráhy, v nichž mají stěžejní úlohu mitochondrie. Spuštění vnější a vnitřní apoptotické signalizace vede k aktivaci proteáz (kaspáz), nukleáz a nakonec k řízenému zničení zasažené buňky. Druhá část práce je zaměřena na podrobný popis apoptotické signalizace navozené ligandem TRAIL a odolností nádorových buněk k TRAILem způsobené apoptóze, jakožto závažné překážce v TRAILem podporované nádorové terapii.

2. Key words

resistance, apoptosis, cytokines, TRAIL, death receptors, tumor, chemotherapy, cancer stem cells

3. List of abbreviations

aa	amino acid
ADD	addiction/dependence domains
AIF	apoptosis inducing factor
Akt	serine/threonine kinase (protein kinase B)
Apaf-1	apoptosis protease activating factor-1
Apo-2	death receptor 4/TRAIL receptor 1
ATP	adenosine 5'-triphosphate
B cell	B lymhocyte
Bad,	Bcl-2 antagonist of cell death
Bak	Bcl-2 antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	apoptosis regulatory protein discovered in B-cell lymphoma
Bcl-x_L	apoptosis regulatory protein allied to Bcl-2
BH	Bcl-2 homology domain
Bid	BH3-interacting domain death agonist
Bik	Bcl-2-interacting killer
Bim	Bcl-2-like 11 protein
Bmf	Bcl-2-modifying factor
Bok	Bcl-2-related ovarian killer
C-terminus	carboxy-terminus/COOH-terminus
Ca^{2+}	calcium cation
CARD	caspase recruitment domain
CED-3	*Caenorhabditis Elegans* cell death-associated protein-3
CKI	casein kinase I
CKII	casein kinase II
CMV	*Cytomegalovirus*
Colo357	pancreatic adenocarcinoma cell line
CRD	cysteine-rich domain
CrmA	*Cowpox virus* serine proteinaise inhibitor (serpin)
CTL	cytotoxic lymphocyte
Cys	cysteine
cyt c	cytochrome c
Da; kDa	Dalton (molecular mass unit 1Da ≈ 1.6605×10^{-27}kg); kiloDalton
dATP	deoxyadenosine 5'-triphosphate
DC	dendritic cell
DCC	deleted in colorectal cancer/netrin-1 receptor
DcR	decoy receptor
DD	death domain
DED	death effector domain
DIABLO	direct IAP-binding protein with low pI/murine homolog of Smac
DISC	Death-Inducing Signaling Complex
DNA	deoxyribonucleic acid
DP5	see Hrk
DR	death receptor

ECM	extracellular matrix
e.g.	for example
ERK	extracellular signal-regulated kinase
EST	expressed sequence tag
etc.	et cetera
FADD	Fas-associated death domain (adaptor protein)
FasL	Fas ligand
FLICE	FADD homologous ICE-like protease/caspase-8
FLIP	FLICE-like inhibitory protein
GDNF	glial cell line-derived neurotrophic factor
GPI	glycosylphosphatidylinositol
Hek-293	human embryonal kidney cell line
His	histidine
Hrk	hara-kiri/Bcl-2-interacting protein/DP 5
HSP90	heat shock protein 90
i.a.	inter alia
IAP	inhibitor of apoptosis protein
ICE	caspase-1
IFN	interferon
IKDC	IFN-γ-producing killer dendritic cell
Il-2	interleukin-2
IR	ionizing radiation
JNK	Jun N-terminal kinase
LIT	decoy receptor 1
LPS	lipopolysaccharide
LT-α	lymphotoxin-α
LTP	long term potentiation
mAb	monoclonal antibody
MAP	mitogen-activated protein kinase
Mcl-1	myeloid cell leukemia protein-1
mDR-5	murine death receptor-5
MOMP	mitochondrial outer membrane permeabilization
mRNA	messenger ribonucleic acid
mTRAIL	murine Tumor-necrosis factor-Related Apoptosis Inducing Ligand
N-terminus	amino-terminus/NH2-terminus
N-DYKDDDDK-C	D = aspartic acid, Y = tyrosine, K = lysisne
NF-κB	nuclear factor κB
NK; NKT cells	natural killer cells; natural killer T cells
Noxa	BH3-only member of the Bcl-2 family
OPG	osteoprotegerin
P35	baculoviral serine proteinaise inhibitor (serpin)
p53	transformation-related protein 53
PCD	programmed cell death
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLAD	pre-ligand assembly domain

Puma	p53-upregulated modulator of apoptosis
RANK	receptor activator of NFκB
RANKL	receptor activator of NFκB ligand
RET	rearranged dring transfection protooncogene
RIP	receptor-interacting protein
ROS	reactive oxygene species
Smac	second mitochondria-derived activator of caspase
SRP	signal recognition particle
T cell	T lymphocyte
tBid	truncated form of BH3-interacting domain death agonist
T_H1	helper T lymphocyte type 1
T_H2	helper T lymphocyte type 2
THD	TNF-homology domain
TNF	Tumor-necrosis factor
TNFR	Tumor-necrosis factor receptor
TNFSF 10	Tumor-necrosis factor superfamily member 10
TRADD	Tumor-necrosis factor receptor-associated death domain protein
TRAIL	Tumor-necrosis factor-Related Apoptosis Inducing Ligand
TRAIL-R	Tumor-necrosis factor-Related Apoptosis Inducing Ligand receptor
TRICK2	death receptor 5
TRID	decoy receptor 1
TRUNDD	decoy receptor 2
WM9	human melanoma cell line
WM739	human melanoma cell line
WM1205	human melanoma cell line
XIAP	X-linked inhibitor of apoptosis protein

4. Introduction

According to the actual World Cancer Report published by World Health Organization with more than 10 million new cases every year, cancer has become one of the most devastating diseases worldwide. Cancer affects everyone the rich and poor, the young and old, men women and children and represents appalling burden on patients, families and societies. Furthermore, the global burden of cancer continues to increase. In the year 2000, 6.2 million died from the disease; the number is expected to grow by 50% over the next 20 years to reach 15 million by 2020. Therefore we have objective reason for developing new strategies to fight against this devastating disease.

Today, possibilities of cancer treatment consist of surgery, radiotherapy, chemotherapy, palliative care and their combinations. All these approaches have been upgraded in order to increase effectiveness and mainly reduce their biggest disadvantage-serious side effects. Regarding chemotherapy, the idea of a "golden bullet" represents the dream of oncologist so far. The golden bullet is hypothetic agent that would exclusively destroy transformed cells without any toxicity towards normal cells. One promising candidate of this concept is TRAIL. This recently discovered molecule is human endogenous ligand expressed mainly on hematopoietic cells. Its capability of inducing programmed cell death and certain degree of selectivity could be very valuable in designing either completely novel type of anti-cancer therapy based on the recombinant ligand, on agonistic antibodies against its pro-apoptotic receptors or on its combination with the current therapeutical approaches. Since its discovery in 1995, TRAIL has been intensively studied, but many questions related to regulation and function of TRAIL-induced signaling remain still unanswered. The following chapters summarize current knowledge TRAIL signaling pathways with a focus on their regulation and importance in tumor biology.

5. Programmed cell death and its significance in organism

Programmed cell death (PCD) is an evolutionary conserved process that is in multicellular metazoans and plants required for elimination no-longer needed or dangerous

(e.g., infected or malignant) cells and for the maintenance of cellular homeostasis (1). PCD relies on firmly regulated cascades of finely-tuned events that finally lead to organized destruction of the dying cell (2). PCD is essential in development (shaping of tissues and organs, e.g., removal of interdigital cells), neuronal network maturation, removal of self-reactive or ineffective cells of the immune system and for other physiological processes (3-5).

PCD can be basically divided into two types- type I PCD or apoptosis and type II PCD or autophagy.(6). Apart from these fundamental types of death, cells can demise via mitotic catastrophe, programmed necrosis or necroptosis and senescence - an irreversible exit from the cell cycle. Apoptosis is the most investigated and best characterized process of the cellular suicide and will be discussed in this work in more details. In contrast to apoptosis, necrosis is entirely different type of cell death. Necrosis is usually connected with cell death via rough and violent treatment (intensive heat, pressure, hypoxia etc.) that leads to cell swelling, cytoplasmic membrane rupture and destruction. It was originally widely recognized that this process can be neither programmed nor controlled. However, several lines of evidence suggest that necrosis can be also considered as PCD since anti-apoptotic signaling as Bcl-2 overexpression can protect cells from both necrotic and apoptotic death (7, 8). Thus necrosis could occur either as accidental, uncoordinated cell death or necrosis-like PCD. Various types of PCD are often combined (9) and distinction between types I, II and necrosis can be therefore rather complicated, hence nomenclature of the cell death has not been unified.

Diverse cell death programs enable cells to response to the developmental processes, environmental changes, and to variable physiological or pathological situations. Deregulation PCD is known to play important role in serious human diseases as cancer, pathological neurodegeneration or autoimmunity (10).

5.1 Apoptosis

The word apoptosis, which originates from Greek, denotes “falling off” or “defoliating” of trees and was introduced by J. F. Kerr in 1972 to describe a specific

morphological pattern of cell death occurring during embryonic development, cell turnover in healthy adult tissue and atrophy upon hormone withdrawal (11). Although terms programmed cell death and apoptosis are often used interchangeably, apoptosis, as mentioned above, represents just one subtype of the programmed cell death (11).

5.1.1 General features of apoptosis

The general morphology associated with apoptotic cell is generally characterized by nuclear condensation, cell and organelles shrinkage, detachment of the apoptotic cell/-s from the neighbouring cells or cell matrix, membrane blebbing and cellular fragmentation into membrane wrapped cellular fragments called the apoptotic bodies. These remnants of cell are then eliminated via phagocytosis by surrounding cells or by professional phagocytes as macrophages. Apoptosis has got over necrotic cell death several distinct advantages. Necrotic cell releases through its disrupted cytoplasmic membrane many substances (e.g., proteases, hydrogen peroxide, nucleases) which are potentially very toxic and dangerous for adjacent cells. This leakage of cellular contents incites massive inflammation of the surrounding tissues. Unlike necrosis, apoptosis is an active process that requires energy of ATP and usually does not induce inflammation. Biochemical signs accompanying apoptosis further include chromatin internucleosomal fragmentation, increase of cytosolic Ca^{2+} concentration, cell surface phosphatidylserine exposure, actin cytoskeleton depolymerization and ongoing protein synthesis (6).

5.1.2 Caspases as apoptosis executioners

Caspases are sit-specific proteases, which cleave tetrapeptide recognition motif in the target proteins after aspartate residue. At present are known at least 14 mammalian caspases and majority of them actively participates in the induction and progression of apoptosis. Some caspases such as caspase-1/-4/-5/-11 are mainly involved in non-apoptotic processes such as pro-inflammatory cytokine maturation and associated anti-microbial inflammatory response. Caspases are expressed as inactive zymogens and require proteolytic processing and dimerization for their full activation. Active caspases contain

cysteine in their active center and form heterotetramer containing two large (20kDa) and two small (10kDa) subunits (12, 13).

Initiator or "priming" caspases are activated in specific multiprotein complexes after recruitment via their characteristic long N-terminal prodomains containing either the death effector domain (DED) (e.g., caspase-8/-10) or the caspase recruitment domain (CARD) (e.g., caspase 9). Upon activation, the initiator caspases cleave effector or "downstream" caspases (e.g., caspase-3/-6/-7) or other proteins at their specific aspartate-containing sites. This processing and following conformational change-induced activation of downstream caspases then unlashes cleavage of wide spectrum of vital proteins as cytoskeletal components (actin, lamins), cell-cycle regulators, anti-apoptotic proteins, inhibitors of nucleases, components of DNA synthesis/repair machinery, kinases etc. (at present over one hundred caspase substrates are described) and subsequent dismantle of cell from within (14). Besides initiator caspases, serine protease granzyme B expressed by natural killer (NK) cells and cytotoxic lymphocytes (CTL) can activate effector caspases to promote apoptosis of virally infected cells (15).

Since caspases function as apoptosis executors, they are potentially dangerous and must be tightly regulated to prevent undesirable cell death. Moreover, apoptotic caspases are also to some extent activated during non-apoptotic cellular events (e.g., differentiation, proliferation (16) or even long term potentiation (LTP) of neural cells (17). Caspase activity is therefore in these processes as well as during apoptosis controlled by cellular inhibitors as caspase-8 competitor FLIP or by inhibitors of apoptosis proteins (IAPs) as well as during viral infection by viral inhibitors called serpins (e.g., CrmA, P35) (13).

5.1.3 Mitochondria and intrinsic apoptotic signaling

Signaling pathways leading to apoptosis are usually divided according to their triggers, which are either extracellular or intracellular (Fig. 2). Apoptosis is, in general, the ultimate reaction to cellular stress, that reflects long-lasting highly unfavourable intracellular conditions (e.g., starvation, heat-shock response, hypoxia, incorrect protein folding). It also serves as primary defense against transformation and viral or bacterial infection. In these potentially very dangerous situations it seems to be profitable for an organism as a whole to sacrifice individual damaged or infected cells (10). The main

activators of the intrinsic apoptotic signaling are reactive oxygen species (ROS), misfolded proteins or damaged DNA, viral infection, and oncogenes (18, 19).

In 1992 anti-apoptotic protein Bcl-2 was described as a protein associated with the outer mitochondrial membrane by Monaghan *et al*. (20). Several years later experiments with cell-free extracts provided evidence that mitochondria play crucial role in apoptosis. In these experiments, in the *Xenopus laevis* egg cell-free extract the apoptotic processes could be activated only when these extracts were enriched with mitochondria (21, 22). These organelles stand on the crossroad of multiple apoptotic signaling pathways that serve as an integrator of largely intrinsic but also some extrinsic signals (Fig. 2) (1).

In mitochondria reside molecules that can promote cell death in either caspase-dependent (cytochrome c) or caspase-independent manner (apoptosis-inducing factor (AIF)) (23). Cytochrome c (cyt c) is soluble 12kDa protein located in intermembrane space of mitochondria serving as a part of electron transport chain. When released, it forms heptameric structure together with caspase-9, dATP and Apoptotic protease activation factor-1 (Apaf-1), so called apoptosome (24). In this multiprotein complex, caspase-9 undergoes activation by proteolytic cleavage and subsequently activates processing of effector caspases and caspase-dependent apoptosis. In mitochondria reside also other pro-apoptotic molecules as IAP inhibitors Smac/DIABLO and HrtaA2/Omi or nucleases AIF or endonuclease G that can apparently promote PCD without activation of downstream caspases (23).

The principal regulators and promoters of apoptosis connected with mitochondria are proteins of the Bcl-2 (discovered in B-cell lymphoma) family (25). This relatively large group of signaling molecules consists of both pro- and anti-apoptotic proteins, whose common feature is presence of one to four so-called Bcl-2 homology (BH) domains (Fig.1).

Bax, Bak and Bok are members of the Bax-like subfamily of pro-apoptotic proteins and contain BH1, 2 and 3 domains. In inactive form Bax resides in cytosol but Bak is already located at the mitochondria and their localization/activity dramatically changes after an apoptotic stimulus. Bax moves to mitochondria where it similarly as Bak undergoes structural changes and via multimerization Bax/Bak produce large pores in the outer mitochondrial membrane, leading to mitochondrial outer membrane permeabilization (MOMP) and release of the pro-apoptotic proteins as cytochrome c or AIF from the

intermembrane space. Bax and Bak can functionally substitute each other and therefore $Bax^{-/-}$ $Bak^{+/+}$ or $Bax^{+/+}$ $Bak^{-/-}$ mice survived with only mild disorders. However, $Bax^{-/-}$ $Bak^{/-}$ mice suffered from serious developmental malfunctions, which could be explained by PCD insufficiency during the embryonic development and hematopoesis. Bax levels in some tumors correlate with efficacy of chemotherapy and human colorectal carcinoma with mutated Bax is resistant to 5-fluorouracil (1).

In contrast to Bax proteins most of the anti-apoptotic Bcl-2 proteins as Bcl-2, Bcl-x_L, Mcl-1, Bcl-W, Bcl-G contain one additional BH4 domain. Major function of these anti-apoptotic proteins is dimerization-mediated inhibition of the pro-apoptotic, pore-forming activities of Bax proteins. Bcl-x_L moreover negatively regulates Apaf-1 and apoptosome formation. Higher Bcl-2 and Bcl-x_L level are also associated with tumor resistance to chemotherapy (26). Interestingly, Del Bello *et al.* 2001 showed that caspase-mediated cleavage of Bcl-2 changes its function from anti-apoptotic to pro-apoptotic allowing smooth progression of the apoptotic process (27).

Finally, BH3-only proteins represent the third subgroup of the Bcl-2 family. These proteins contain only BH3 domain and function as sensors or sentinels of cellular stress (28). Role of BH3-only proteins was first elucidated from experiments with nematode worm *Caenorhabditis elegans*, which is widely used as a model organism also for the apoptosis research. In this worm signaling pathways leading to apoptosis are very straightforward and only single representative from each class of the key regulators is known. Its effector caspase CED-3 is activated in complex with Apaf-1 homolog CED-4. CED-4 is suppressed by the anti-apoptotic Bcl-2 homologue CED-9. Apoptosis occurs when BH3-only homologue Egl-1 binds to CED-9 and thus prevents CED-4 inhibition (28). In mammalian cells apoptotic signaling activated by BH3-only proteins is by far more complex and at the present there is known over ten BH3-only proteins (e.g., Bim, Bid, Bad, Bik, Noxa, Puma, Hrk/DP5, Bmf). Individual BH3-only proteins are responsible for apoptotic responses to various kinds of cellular stress and are activated through posttranslational modification (Bim, Bad, Bmf), *de-novo* transcription (Puma, Noxa, Hrk) or by proteolytic processing as Bid. Activated BH3-only sentinels then translocate to mitochondria where they bind to and inactivate anti-apoptotic, Bax-inhibitory function of the Bcl-2 proteins (28).

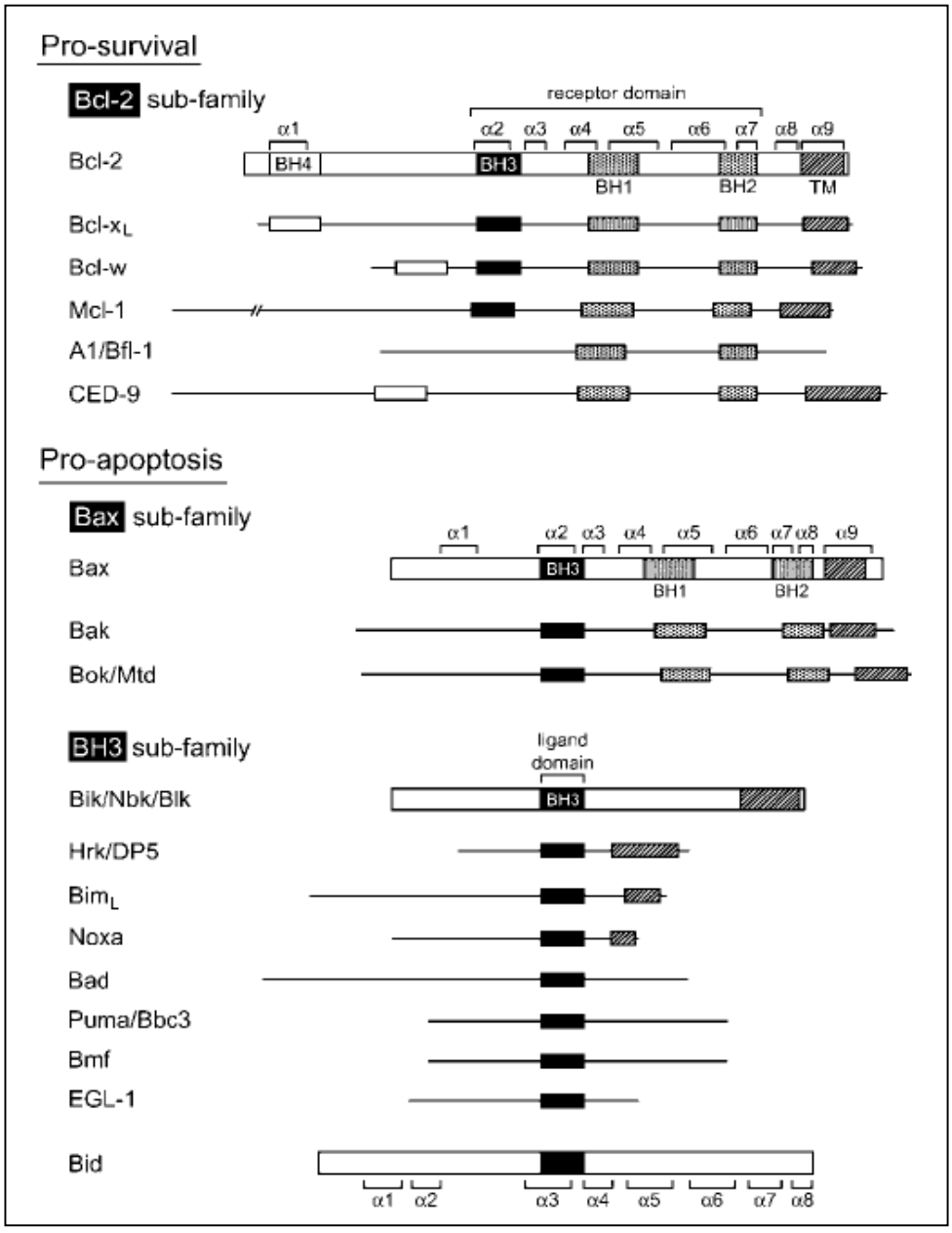

Figure 1 Three subfamilies of Bcl-2 related proteins; ad ref. (25).

5.1.4 Extrinsic apoptotic signaling

Extrinsic apoptotic pathways are mainly activated by transmembrane "death receptors" of the TNFR family as well as by dependence receptors or via integrins (lost of the extracellular contact - anoikis) (29).

Death receptors (Fas/CD95, TRAIL receptors DR4 and DR5, TNFR1, DR3 or DR6) contain specific protein-interaction domain named death domain (DD) in their intracellular parts (Fig. 3). They are similarly as their ligands trimeric transmembrane proteins and their expression is mainly restricted to vertebrates. Upon crosslinking with their ligands and under appropriate cellular conditions, most of these receptors induce caspase-dependent apoptosis of affected cells (30).

Ligand-activated receptor form intermediate activation complexes that in the apoptotic branch of their intracellular signaling contain the adapter protein FADD. TNFR1 recruits FADD indirectly via another adaptor called TNF receptor-associated death domain (TRADD). FADD in addition to receptor-interacting DD also contains a death effector domain (DED), which serves for subsequent recruitment of procaspase-8 and formation of Death-Inducing Signaling Complex (DISC). DISC, similarly as apoptosome, serves for proximity-induced initiator caspases-8/-10 processing and activation. At the level of DISC apoptosis can be suppressed by competitive inhibitor FLICE-like inhibitory protein (FLIP), a protein similar to caspase-8 but lacking protease activity. Active caspase-8 then cleaves its downstream targets as effector caspases or the BH3-only sentinel Bid. tBid then translocates to mitochondria where inhibits anti-apoptotic proteins and thus enables Bax/Bak-mediated MOMP and apoptosis (26). Upon caspase inhibition can DR as Fas or TNFR1 also promote cell death resembling necrosis called necroptosis via RIP1-activated signaling (31).

Dependence receptors form a group of structurally diverse mainly transmembrane proteins that activate apoptosis not in the presence but in the absence of their appropriate ligand. In contrast, ligated receptors usually participate in proliferation or differentiation-related signaling. Physiological roles of this recently distinguished, non-homogenous group are not fully elucidated - some of them as netrin-1 receptor DCC or GDNF receptor RET are essential for development of the neural system (32). Several dependence receptors are also mutated in cancer cells, for example DCC ("Deleted in Colorectal Cancer") is often

deleted in familiar colorectal carcinomas. Most of the dependence receptors contain in their intracellular parts distinct domain named addiction/dependence domain (ADD). Proteolytic cleavage of ADD by caspases (eventually by other protease) leads to production (release or exposal) of pro-apoptotic ADD fragments capable of activating caspase-dependent apoptosis. Ligand binding could as shown for the androgen receptor disable receptor processing (29).

Anoikis or homelessness is form of cell death induced by loss of the contact between extracellular matrix (ECM) and integrins, and in this way it resembles dependence receptors-induced apoptosis. Integrins mediate through their transmembrane heterodimers of α and β subunits cellular adhesion to ECM. Loss of the integrin-mediated contact to ECM suppresses integrins-activated pro-survival signaling mediated by PI3K/Akt and MEK/Erk kinases and consequently leads to caspase-dependent apoptosis induced mainly by BH3-only sentinels as Bad or Bmf. Anoikis represents one of the first defences against cellular transformation and metastatic dissemination of primary tumors (33).

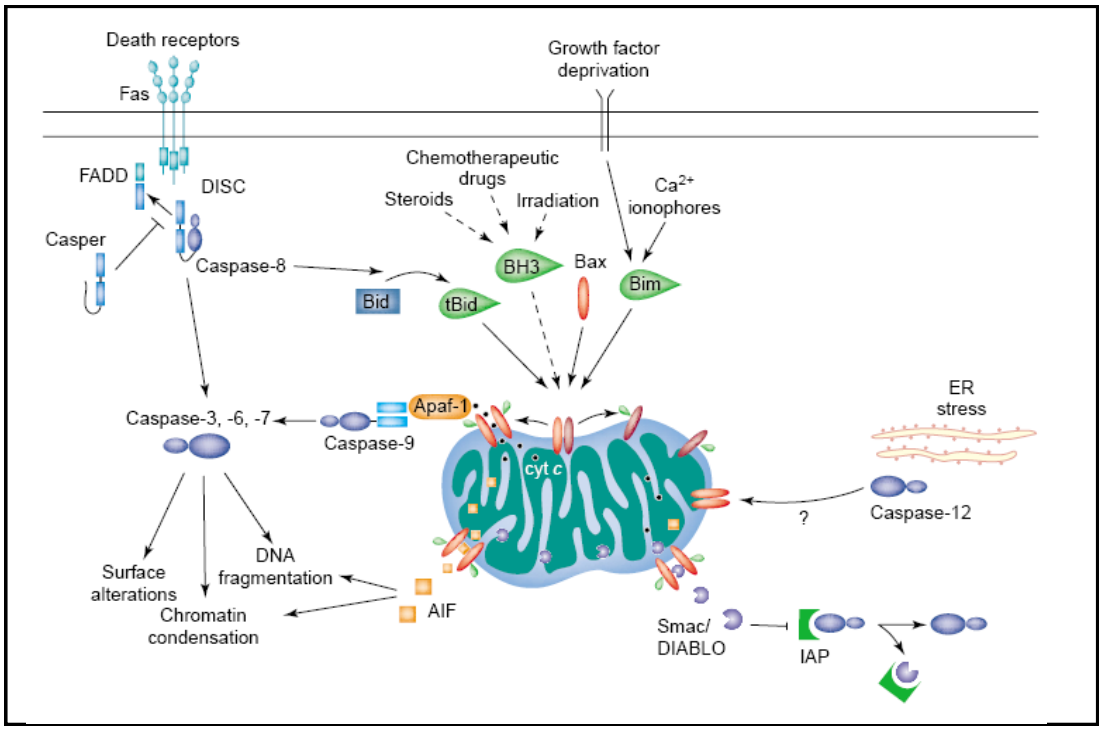

Figure 2. Overview of apoptotic signaling in mammalian cell; ad ref. (1).

6. TNF and TNFR superfamily

The tumor-necrosis factor (TNF) was initially described by P. Burns in 1868, as an unknown agent that mediated tumor-necrotic activity in mice and this activity was induced by concurrent bacterial infection (30). In 1968 this necrotic activity was assigned to a 17kDa protein produced by macrophages, and named lymphotoxin (LT) and later tumor-necrosis factor-β (TNF-β). Then many other members of this newly established family containg TNF structural motif – TNF Homology Domain (THD) were described, and at present the family consists of 19 members that activate diverse cellular processes as proliferation, differentiation and apoptosis (Fig. 3) (34). THD contains approx. 150 aromatic and hydrophobic amino shares about 30% sequence homology among TNF family members. Their cognate receptors, TNF receptor (TNFR) superfamily, consists of 29 members and are distinguished by presence of cysteine bridges-containing pseudorepeats, also called cysteine rich domain (CRD) in their extracellular parts. As mentioned in the previous chapter, a subgroup of the receptors within TNFR superfamily contains a death domain (DD) and can upon activation induce apoptosis of the receptor expressing cells. Another subgroup is known for absence of signaling ability, so they were denoted as "decoy receptors". Rest of known TNFR superfamily members participate in mediating cellular proliferation and differentiation, yet some receptors' ligands are still missing e.g., for receptor expressed in lymphoid tissues (RELT, DR6 or TROY) (30).

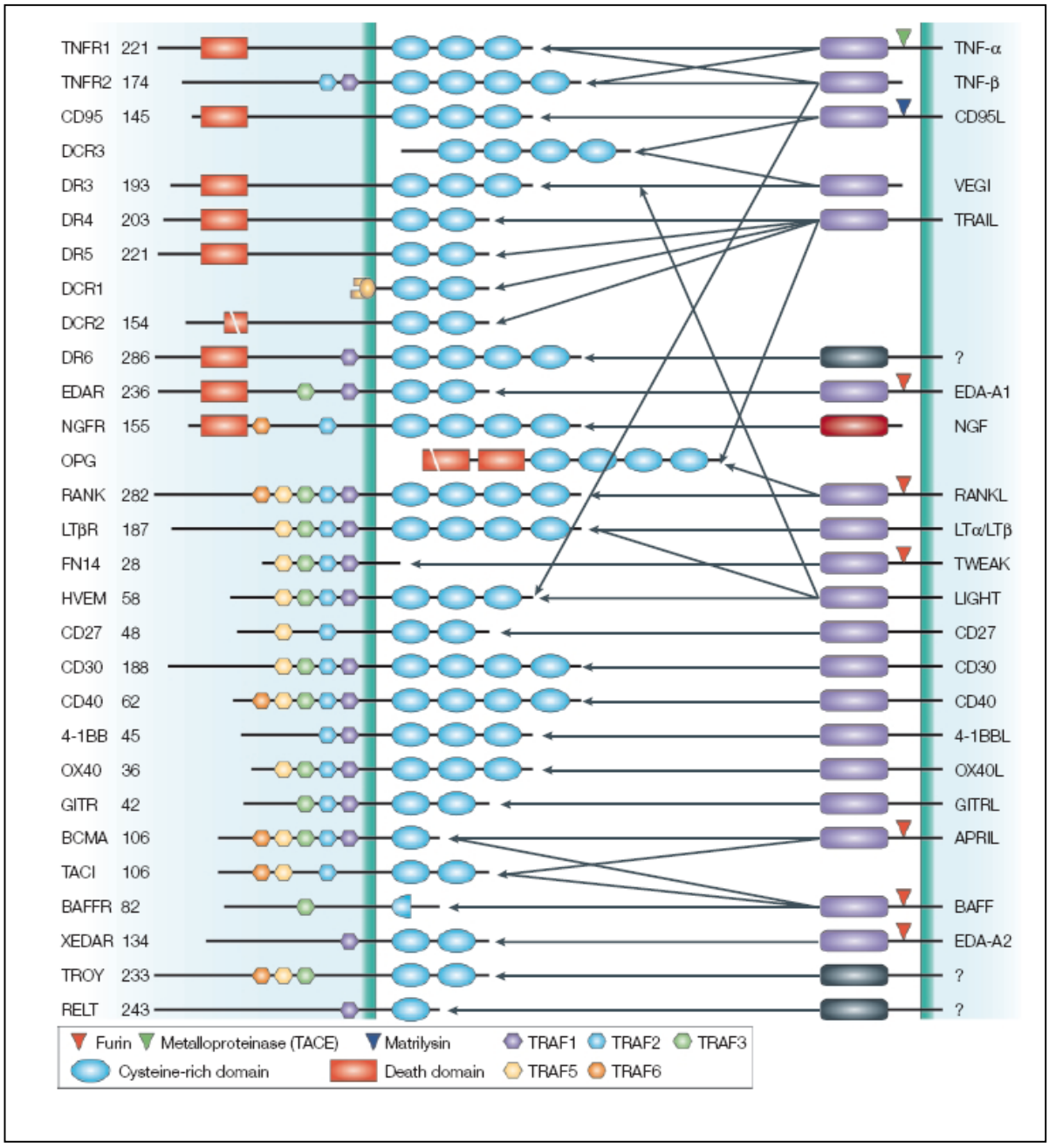

Figure 3. A diagrammatic representation of ligands of the TNF superfamily and their receptors; ad ref. (30).

7. TNF-Related Apoptosis Inducing Ligand (TRAIL) and its role in tumorigenesis

7.1 Introduction to TRAIL

TRAIL or Apo2 ligand (TRAIL/Apo2L/TNFSF10) is a cytokine of the TNF superfamily capable of inducing cell death via its cognate death receptors. It was discovered in 1995 by THD homology-based computer search in expressed a sequence tag (EST) library (35). Since discovery of its specific anti-tumor apoptosis-inducing properties TRAIL has been subject of an intense research as a potential anti-cancer agent. Over past twelve years, many research groups have been trying to elucidate detailed physical and biochemical properties of TRAIL, its physiological function and first of all, its role in anti-tumor defense or regulation of the immune system. Hand in hand with advances in molecular biology of cellular signaling as a whole, better explanation of signaling pathways induced by TRAIL form a scaffold for new approaches of rational anti-cancer or other therapies.

7.2 Structural and biochemical characterization of TRAIL

Human gene for TRAIL, is localized at 3q26 of chromosome 3 and encodes a 281aa protein with predicted molecular weight 32509Da (35). TRAIL is a typical type II transmembrane protein, with one transmembrane hydrophobic region and absent signal sequence. Although TRAIL occurs mostly in membrane bound form, it can be also proteolytically cleaved, albeit apoptosis-inducing activity of this soluble form is compromised (36). As other members of the TNF superfamily, TRAIL contains THD and forms “bell-shaped” trimers (Fig. 4). Common features of THDs are a very similar tertiary fold and their ability to form trimeric proteins (36). Crystallographic studies revealed that each subunit contains a rigid frame of two anti-parallel beta sheets (beta sheet sandwich structure) and loops connecting individual sheets are highly disordered in the thinner part

of the bell. TRAIL is unique within the TNF superfamily as it contains a 15 residue-long extension in one loop that spans the complete outer surface of the monomer (37). This elongated loop is suggested to enable specific recognition of TRAIL receptors (37). Another exceptional feature of the TRAIL is presence of zinc ion binding site made of cysteine (Cys)-230 residues at the trimer interface, which is crucial for the maintenance of optimal biological activity (38). Preparations of soluble recombinant TRAIL lacking zinc display reduced solubility, activity and tend to aggregate. This could possibly explain earlier reported toxicity of certain TRAIL preparations to human hepatocytes (39), since in another study untagged trimeric TRAIL containing zinc was apparently non-toxic (40).

For its specific tumor cells-induced apoptosis TRAIL received significant attention as potentially novel anti-tumor agent. Several versions of the recombinant TRAIL (portion of the extracellular part expressed in an heterologous system) with slightly different biological activities were prepared and tested in various systems. They are non-tagged, soluble and native TRAIL (amino acids 114–281), polyhistidine (His)-tagged recombinant soluble TRAIL (amino acids 95–281), recombinant soluble TRAIL fused to a trimerizing leucine zipper (amino acids 95–281) and FLAG-tagged soluble native TRAIL (41). FLAG-tag (octapeptide: N-DYKDDDDK-C) same as His-tag are added to TRAIL for its more effective isolation by affinity chromatography.

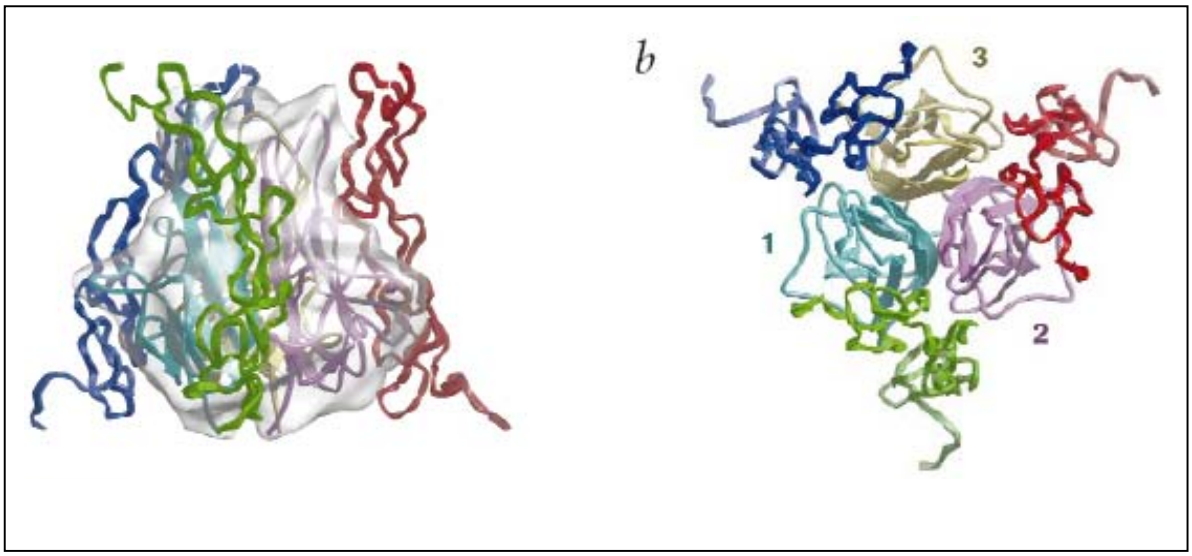

Figure 4 The structure of the TRAIL-DR5 complex. Left: trimer of TRAIL subunits (yellow, cyan, pink) and DR5 (blue, green, red); TRAIL trimer is enclosed in a transparent molecular envelope. Right: the comlex wieved down the three fold axis; ad Mongkolsapaya, 1999.

7.3 TRAIL Receptors

TRAIL interacts with five receptors, but only two of them can transmit apoptotic signaling. As the first human TRAIL receptor was identified death receptor 4 or TRAIL receptor 1 (DR4/TRAIL-R1) in 1997 (42). Surprisingly, almost simultaneously were by EST database search discovered remaining four TRAIL receptors (one pro-apoptotic and three inhibitory) (30). Their discovery opened yet not resolved discussion about a purpose of their multiplicity.

Two of the five TRAIL receptors, namely DR4/TRAIL-R1 and DR5/TRAIL-R2 (also called Killer and TRICK2) are capable of inducing cell death after TRAIL ligation. Death receptor 4 has two CRD while death receptor 5 has one partial CRD (with only one out of three usual cysteine bonds) and two full CRD and shares 58% homology with DR4. Moreover, DR5 has two alternative splicing forms dubbed DR5A and 23aa longer DR5B; however, no difference in their functions has been reported (43, 44). Both receptors are expressed in wide range of tissues and are strongly upregulated on activated lymphocytes (43).

Similarly as other death receptors (TNFR1, Fas) TRAIL receptors apparently form pre-assembled trimers via novel pre-ligand assembly domain (PLAD) (45). High degree of homology in PLAD would allow TRAIL receptors to form heterotrimers allowing thus another level of regulation of TRAIL-induced apoptosis. Indeed recently published report documents apoptosis-inhibitory heterotrimerization between DR5 and TRAIL-R4 (46).

As TRAIL signaling is proposed to play an important role in tumor immune surveillance mutations in components of TRAIL signaling pathway can occur during tumorigenesis. Polymorphism in the ligand binding domain of DR4 has been connected with a higher risk of bladder cancer, and other mutations within DR4 have been reported in cancer cells (47). Conversely, the expression of non-mutant DR4 in colon tumors was linked to favourable prognosis (48). Expression of both DR4 and DR5 is also upregulated

in p53-dependent manner in drugs or ionizing radiation treated tumor cells, pointing to interdependence of intrinsic and extrinsic apoptotic signaling (49-51).

Existence of two death receptors for the same ligand could indicate their distinct role in regulation of tissue homeostasis or activation of secondary signaling pathways. In support of their distinct roles, Muehlenbeck *et al.* using DR5-specific TRAIL mutants showed that the JNK pathway is preferentially activated by DR5 (52). Colon cancer cells preferentially use DR5-triggered apoptosis (53) and in contrast chronic B cell leukemia cells activate upon TRAIL ligation DR4-dependent apoptosis (54). Also their transport to the cell surface is differently regulated as knockdown of signal recognition particle (SRP) protein SRP72 compromises only DR4 but DR5 cell surface localization (55). Increased DR5 expression could be characteristic for transformed cells as majority of normal cells express only low levels of DR5 at the cell surface (53).

In addition to DR4 and DR5, TRAIL interacts with three other so-called decoy receptors - their ligation with TRAIL does not induce cell death. The first described TRAIL decoy receptor was decoy receptor 1 (DcR1/LIT/TRID/TRAIL-R3). Unlike the other receptors, TRAIL-R3 lacks the cytoplasmic part and is attached to the membrane through a glycosyl-phosphatidilinositol (GPI) anchor. It is less widely expressed than other TRAIL receptors (56). Decoy receptor 2 (DcR2/TRUNDD/TRAIL-R4) is relatively more expressed than TRAIL-R3 and has got a high degree of homology to the other TRAIL receptors. It has transmembrane region and a truncated death domain lacking two thirds of its regular length (57). This truncated DD is incapable of initiating cell death, probably due to a failure in DISC formation. The initial interpretation of their function was that they could serve as “decoys” competing with DR4/5 for binding to TRAIL. Nevertheless, this “decoy hypothesis” was challenged after experiments using monoclonal antibodies specific for each of the membrane-bound TRAIL receptors found no correlation between TRAIL resistance and decoy receptors expression. However, several new lines of evidence recently implied that fore mentioned receptors actually act as effective DR antagonists at least in some tissues, thus the “decoy hypothesis” is not absolutely refuted. Moreover some other hypotheses of antagonistic functions for these receptors have been recently proposed and will be discussed in more details in the chapter about TRAIL resistance. Third

antagonistic TRAIL receptor is not membrane bound but soluble osteoprotegerin (OPG). OPG is also high affinity decoy receptor for RANKL (receptor activator of NF-κB ligand) another ligand of the TNF family, which plays an important role in bone metabolism. TRAIL interacts with OPG less efficiently than RANKL (58) and thus it is not clear, how important would be TRAIL-OPG interaction in the regard to the suppression of TRAIL-induced apoptosis or to bone metabolism. However, no functional relation between RANK-RANKL and TRAIL-TRAIL-R systems has been reported up to date.

7.4 TRAIL-induced signaling pathways

The main signaling TRAIL receptors are TRAIL-R1/DR4 and TRAIL-R2/DR5 and very little known about possible signal transduction of decoy receptors (59). TRAIL death receptor signaling does not have to induce apoptosis by default, but under certain conditions when apoptosis is inhibited it also induces proliferation of the target cells (60).

TRAIL-induced apoptotic signaling resembles Fas-induced apoptosis. Ligation of DR4 and DR5 by trimeric TRAIL leads to assembly of DISC composed of the adaptor protein FADD and procaspase-8 and/or -10. The TRAIL receptor basal unit is a trimer, but it is likely that multiple trimers are being activated together to form functional DISC and aggregation of death receptors in large groups is probably required for effective apoptotic signaling. Proximity-activated processing of the initiator procaspases leads to their full activation and unlashing the downstream signaling that eventually activates the effector caspases and cell death. DR4 and DR5 are also similarly as Fas associated with lipid rafts and their interaction with lipid rafts enhances their apoptosis-inducing potential (61).

In analogy with Fas, the TRAIL-induced apoptotic signaling can be divided into two groups. In type I cells, caspase-8 is activated at the DISC in quantities large enough to directly activate effector caspases as caspase-3. In type II cells, however, the amount of active caspase-8 produced at the DISC is smaller and insufficient for effective activation of the effector caspases. In these cells a mitochondrial amplification loop is required for full activation of caspases. This is achieved by cleavage of a Bcl-2 family member Bid. When cleaved by caspase-8, Bid translocates to the mitochondria, where it together with Bax/Bak participates in activation of the mitochondrial apoptotic signaling (26). Differences

between type I and type II cells can have important implications for cancer cell resistance mechanisms.

Apoptotic signaling from TRAIL death receptors is default signaling pathway at least in the transformed cells, but activated TRAIL receptors can also trigger other signaling pathways as ERK, JNK, p38 MAP kinase and NF-κB ones (60). Some of these signals are generated by a second cytoplasmic complex (complex II) that forms after the DISC (62). Recent studies imply that complex II, which results from receptor internalization is a common phenomenon in death receptor mediated apoptosis and it may act as a platform for caspase-8 to be further cleaved and fully activated. In addition, recruitment of receptor interacting protein (RIP), TRADD, and IKKα (that serve for NF-κB activation) to the complex II was reported (63). Interestingly, the proteins that are required for apoptosis and NF-κB activation are recruited competitively (62). In TRAIL-resistant tumor cells activation of these secondary signaling pathways (mainly NF-κB) enhances their proliferation potential. Thus, TRAIL binding can promote tumor growth and survival in such cells (60). TRAIL was also reported to enhance expression of pro-inflammatory cytokines in TRAIL-resistant pancreatic cancer cells, and promote thus their metastatic potential (64).

7.5 Physiological function of TRAIL

Physiological in vivo function of TRAIL-induced signaling was intensively studied in murine models. However, instead of two signaling human TRAIL receptors DR4 and DR5, mice have only one named mDR5 (murine death receptor 5), which is similarly homologous to the human DR4 and DR5 (65). Thus the biological function of TRAIL signaling in mice does not have to fully reconcile its function in humans. Several different approaches have been used to assess physiological function of TRAIL in mice: 1) blocking soluble recombinant DR5, 2) genetic inactivation of TRAIL and its mDR5 receptor and 3) neutralizing anti-mouse (m)TRAIL monoclonal antibody (mAb) (66, 67).

$TRAIL^{-/-}$ mice were viable, fertile and without any developmental defects and their examinations did not point to any crucial role for TRAIL in embryonic development (68). However, their enlarged thymus raised former suspicion that TRAIL and its receptors have

a role in the immune system (68). Possibility that TRAIL has a role in immunosurveillance of cancer has been extensively studied after Sedger *et al.* showed that a syngenic transplant of a B cell lymphoma grows much slower in $TRAIL^{+/+}$ mice and also forms less liver metastases than TRAIL-deficient mice (68). Later this anti-metastatic effect was assigned to NK cells and NKT cells that reduced the number of liver metastases in a TRAIL-dependent manner. $TRAIL^{-/-}$ and $TRAIL\text{-}R^{-/-}$ mice displayed higher frequency in developing spontaneous lymphomas at higher age (more than 500 days). Also tumors induced with the carcinogen methyocholantrene developed more often in $TRAIL^{-/-}$ mice (66). However, TRAIL system does not function as a typical tumor suppressor. Loss of TRAIL-R did not accelerate incidence of lymphomas in p53-deficient mice (69). In summary, the TRAIL/TRAIL-R system at least in mice does not overtake of a tumor suppressor but apparently serves as one of the "weapons" of the immune surveillance that could be important for anti-metastatic defense. However, as mentioned in the beginning of this chapter, TRAIL system could play much more important role in anti-tumor protection in long-lived mammals as humans.

In spite of mTRAIL mRNA expression in majority of tissues, there is no detectable mTRAIL on the surface of freshly isolated T cells, natural killer (NK) cells, NKT cells, monocytes, dendritic cells (DC) or B cells (67). Nevertheless many innate immune cells as NK cells express mTRAIL after stimulation with interferon (IFN)-γ, IFN-α, IFN-β, interleukin (Il)-2, Il-15 or lipopolysaccharide (LPS) (70). Subset of hepatic NK cells constitutively expresses TRAIL in an autocrine IFN-γ-dependent manner. Immature NK cells also express TRAIL and lose its expression after their maturation into granulated NK cells , whereas a small NK cells group does not undergo this maturation (71). The persistent TRAIL-positive NK cells subset could then possibly serve for elimination of immature DCs, which are sensitive for TRAIL-induced apoptosis (72).

TRAIL cell surface expression is increased on stimulated T cells. Anti-CD3 mediated activation of T_H1 but not T_H2 leads to upregulation of TRAIL and simultaneous increase of anti-TRAIL resistance in respective cells (73). TRAIL signaling could also play a role in development of autoimmunity. Most of murine model-based studies describe a suppressive function of TRAIL on progress of artificially induced autoimmune diseases

such as encephalomyelitis, diabetes or rheumatoid arthritis (74). Nonetheless, other autoimmune diseases as neuroinflammation may be accelerated by TRAIL (75).

7.6 Regulation of TRAIL signaling

7.6.1 TRAIL receptors-associated resistance

Originally, decoy receptors TRAIL-R3/DcR1 and TRAIL-R4/DcR2 were thought to confer TRAIL resistance of normal and some tumor cells. This presumption came from the fact that DcRs lack functional death domain, and experiments indicating correlation between their expression and resistance to TRAIL-induced killing (30). However, some TRAIL-sensitive cells as melanoma cell lines (WM 793, WM9 and WM1205) express high levels DcR1 and/or DcR2 and are still being sensitive to TRAIL-induced apoptosis (76). Nevertheless, evolutional conservation of the decoy receptors expression together with their high expression of some normal cell (as thymic T cells) argues for their important role in regulation of TRAIL-induced apoptosis. Mérino *et al.* recently proposed a model, in which TRAIL-R3/DcR1 titrates TRAIL within lipid rafts, while TRAIL-R4/DcR2 forms inactive trimers with DR5 (46).

Another source of TRAIL resistance at the receptor level could be their non-functional mutations. A polymorphism in DR4 has been described in gastric adenocarcinoma, head and neck squamous cell cancer and lung cancer. C-to-G alteration at nucleotide 626 of DR4 results in substitution of an arginine for threonine at codon 209 (T209R). Another missense mutation was G-to-A at nucleotide 422 changing histidine for arginine at codon 141 (R141H) (77). Interestingly, these two amino-acid changes occurred in or near the ligand binding domain of DR4, so they could possibly affect receptor trimerisation or ligand binding. DR4 or DR5 mutations were identified in seven out of 34 specimens from breast cancer patients with metastatic disease. Two of the mutations were located within DD and two in the DD flanking region. Mutated DR4 or DR5 when expressed in HEK-293 cells suppressed their TRAIL-induced apoptosis (77). This led to conclusion that DR4/5 may act as tumor suppressor genes in some breast cancer and may loose this function during progression into metastatic stages.

7.6.2 Regulation of TRAIL signaling at DISC

Assembly of the DISC is an essential molecular event in the signaling pathway of TRAIL-induced apoptosis and dysfunction in any of DISC components can lead to TRAIL resistance (78).

The fact that absence of FADD expression was found in different types of tumor cells both in mice and humans suggested that absence of FADD contributed to tumor development. FADD is the main signal transducing intermediate adaptor molecule of several death receptors including Fas, TNFR1, DR3 and TRAIL-Rs (61). Thus one would expect that lack of FADD expression in tumor cells must confer multiple resistance of these cells to death receptor cytotoxicity. In agreement with this assumption, FADD-deficient Jurkat cells were resistant to TRAIL even at very high concentrations (1 μg/ml), in contrast to sensitive wild-type Jurkat controls, which underwent apoptosis at TRAIL concentrations as low as 10 ng/ml (79). In addition, recent experiments revealed non-apoptotic functions of FADD phosphorylated by casein kinase I (CKI). Upon phosphorylation FADD changes its intracellular localization and induces NF-κB and this event has been associated with poor outcome in lung adenocarcinomas (80). It would be worth finding out if this dual role of FADD also affects signal transduction from death receptors.

In 2001, Eggert *et al*. reported frequent loss of caspase-8 in neuroblastoma cells, which significantly rendered these cells TRAIL-resistant. Treatment with methylase inhibitor 5-aza-2'deoxycytidine restored mRNA and protein expression of caspase-8 and TRAIL-sensitivity of resistant cell lines, suggesting that gene methylation was involved in caspase inactivation (81). Loss of caspase-8 expression has further been demonstrated in malignant medulloblastomas, Ewing tumor, rhabdomyosarcomas, retinoblastomas, primitive neuroectodermal brain tumors, and small cell lung carcinomas (82, 83). In many instances, gene hypermethylation has been proposed as the mechanism of silencing caspase-8 gene expression. However, methylation of the region upstream of exon 1 is not associated with caspase-8 silencing (84), suggesting either that DNA methylation of other regions of the gene may affect the expression of human caspase-8 gene indirectly or that other mechanisms of gene inactivation are also present in these tumor cells. In this respect, inactivating caspase-8 gene mutations have also been described in some types of tumor cells such as colorectal carcinoma and squamous carcinoma cells (85). Recently, a distinct

mechanism of caspase-8 suppression has been revealed. McDonald *et al.* isolated a family of apoptotic inhibitors [caspases-8-and-10-associated RING proteins (CARPs)] that bind to and negatively regulate DED containing caspases (86). When overexpressed, CARPs can contribute to the ubiquitin-mediated degradation of DED caspases. Importantly, CARPs overexpression is very often in tumors and tumor cell lines, and their silencing leads to efficient apoptosis and suppression of cancer cell growth.

Competitive inhibitors of procaspase-8, FLIPs (long $FLIP_L$ and short $FLIP_S$ splice variants) can when overexpressed protect cells from death receptor-induced apoptosis. Indeed FLIP overexpression was detected in colonic adenocarcinomas, hepatocellular carcinoma and melanoma (87). Despite findings that overexpression of FLIP increase resistance TRAIL resistance, physiological function of FLIP remains unclear. It was recently suggested that FLIP may help cancer cells to acquire some degree of immune privilege. In experiments with stable transfectants, inoculation of transfectants expressing little FLIP into immunocompetent mice resulted in rejection of the transfectants, but inoculation of transfectants with high FLIP expression into the same type of mice led to tumor development. By contrast, inoculation of either type of transfectants into nude mice led to equal tumor formation and growth, regardless of FLIP expression level. These findings imply that tumor cells with low FLIP expression can be eliminated by immune system, while tumor cells with high FLIP expression can escape immune surveillance (88).

7.6.3 Regulation of TRAIL signaling at mitochondria

Mitochondrial level of apoptosis propagation is mainly important regarding type II cells. As mentioned above these cells need mitochondrial amplification loop for successful propagation of cell death (26). Bcl-2 protein family and mitochondrial inhibitor of IAPs named Smac/DIABLO play the main roles at this level of regulation (Fig. 2).

Retroviral expression of Bcl-x_L in originally TRAIL-sensitive cell line Colo357 rendered these cells TRAIL-resistant (89). Interestingly Bcl-x_L-overexpressing Colo357 cells had the same degree of caspase-8 cleavage as the original TRAIL-sensitive cells suggesting that caspase-8 activation was upstream of the mitochondrial pathway. In another study overexpression of Bcl-2 conferred protection against TRAIL in neuroblastoma and

glioma cell lines (90). In this case TRAIL-induced caspase-8 cleavage was reduced suggesting that caspase-8 was activated both upstream and downstream of mitochondria in these cells. As apoptosis induced by chemotherapy acts mainly through the mitochondrial pathway (26), downregulation of. Bcl-2 and Bcl-x_L might restore sensitivity not only to chemotherapy but also to TRAIL in these types of cancer.

Smac/DIABLO is apoptosis regulator that is normally resident in mitochondria. After mitochondrial apoptotic pathway activation, it is released to the cytosol where it binds IAPs and thus hinders their inhibitory function (1). Blocking the release of Smac/DIABLO has been associated with TRAIL resistance in some, but not all, melanoma cell lines (91). TRAIL-resistant type II cancer cell lines compared with TRAIL-sensitive type II cell lines show reduced release of Smac/DIABLO and overexpression of Smac/DIABLO by transfection sensitized the resistant TRAIL-resistant type II cells to TRAIL. Therefore Smac/DIABLO seems to be one of the major determinants of TRAIL sensitivity in these cells.

7.6.5 TRAIL resistance and kinases

TRAIL-induced apoptotic pathway can be also regulated at the posttranslational level, mainly by phosphorylation of its individual components. A number of kinases as MAP kinases, protein kinase B (PKB/Akt), protein kinase C (PKC), casein kinase I (CKI) and casein kinase (CKII) were shown to affect TRAIL-induced apoptosis.

The MAP kinases are a family of serine/threonine protein kinases that in response to extracellular stimuli regulate variety of cellular activities including cell growth, differentiation, inflammation, and cell death (92). Extracellular signal-regulated kinases 1 and 2 (ERK1/2) activity has been connected with TRAIL-resistant melanoma cell lines (93). Suppression of ERK1/2 activation significantly sensitized these cells to TRAIL-induced apoptosis. However in other cells as lung or colon cancer cells activity of these MAP kinases was required for their more efficient TRAIL-induced apoptosis (94).

Such as MAP kinase pathways, the PI3K-Akt pathway was reported to promote tumor cell survival through upregulation of FLIP, stabilization of XIAP and inactivation of

Bad, Bax and caspase-9 (95). Furthermore, it can activate NF-κB and increase p53 degradation (96). Many reports describe inhibitory role of Akt in TRAIL signaling, whereas downregulation of constitutively active Akt enhanced sensitivity of these cells to TRAIL-induced apoptosis (97).

Protein kinases C constitute a family of serine/threonine kinases with at least ten today known isozymes (e.g., PKCα, PKCγ, PKCδ, PKCε, PKCζ). They participate in a wide set of cellular processes such as cell migration, apoptosis, survival and proliferation. PKCs were originally thought to be pro-mitogenic kinases but this seems to be PKC-isozyme-dependent and cell type-dependent as many PKCs can also inhibit cell-cycle progression (reviewed in (98)). Probably the most striking example of distinct responses conferred by PKC isozymes is the contrasting role of PKCε and PKCδ in apoptosis and survival. PKCδ has been reported to be involved in apoptotic cascade both upstream and downstream of caspase-3, amplifying its activation. Further PKCδ positively regulates intrinsic pathway, as it translocates into mitochondria and enhances cytochrome c release upon apoptotic stimuli. By contrast, PKCε displays mainly anti-apoptotic function. It was shown that PKCε depletion suppressed Akt expression and PKCε overexpression prevents apoptosis induced by TRAIL in glioma cells. Similarly, melanoma cells with low PKCε expression are highly sensitive to TRAIL-induced apoptosis (98). In lung cancer cells PKCε is sufficient to activate ERK and upregulates XIAP and Bcl-x_L (99).

Last but not least, casein kinases have been shown to be important regulators of cell survival with relevant inhibitory effect on death receptor associated pathways. Both casein kinases I and II are conserved serine/threonine kinases present ubiquitously in cells. Initially, the role of CKII in supporting cell proliferation has been described and its common upregulation in cancer cells raised suspicion that it could also inhibit apoptotic pathways. Recent studies by Wang *et al.* provided evidence that CKII overexpression not only promotes suppression of drug-mediated apoptosis, but it stifled apoptosis induced by TRAIL, TNF-α and FasL in their cognate sensitive cells (100). In support of this conclusion were results from another study where rhabdomyosarcoma cells were sensitized to TRAIL-induced apoptosis after CKII inhibition (101). This inhibition resulted in enhanced recruitment of caspase-8 to DISC and caspase-8 mediated cleavage of Bid.

7.7 TRAIL as potential anti-cancer-therapy agent

Members of the TNF superfamily as TNF-α or Fas ligand attracted attention for their tumor-induced apoptosis as potential anti-tumor agents. However, these agents also induced severe hepatotoxicity and sepsis-like symptoms in treated animals and thus were unsuitable for systematic anti-tumor treatment.

In contrast to TNFa or FasL, systematic administration of the recombinant TRAIL did not lead to any toxic side effects neither in mice nor in primates (102). TRAIL is thus being a rare example of such molecules that can eliminate many transformed cells while sparing normal ones. Moreover, TRAIL can induce tumor cells apoptosis independently of their p53 status (103) (tumors with mutated p53 are more difficult to eradicate using the conventional chemotherapy). This offers an attractive possibility of simultaneous administration of TRAIL and widely-used anti-cancer drugs or radiation treatment. Synergistic effects of TRAIL with etoposide, cisplatin, irinotecan oxaliplatin; protease inhibitors MG321 and bortezomib and γ-irradiation on enhancing tumor cells apoptosis have been documented in a number of reports (reviewed in (104)) . The mechanisms in the background of this synergy involve DRs upregulation, enhanced DISC assembly, caspase upregulation and downregulation of IAPs and FLIP. At the present are ongoing phase I/II clinical trials with both recombinant TRAIL (Genentech) and with the agonist humanized anti-TRAIL-R1 and -R2 monoclonal antibodies (Human Genome Sciences) and the up to now results look very promising (104).

8. Conclusions

The last twelve years of research on TRAIL has revealed that this cytokine is truly an interesting molecule with myriad of functions in both immunity and cancer. Although much progress has been made with a view to TRAIL signaling and its crosstalk with other signaling pathways, it seems as if we have only enlightened the tip of the iceberg so far. Thanks to its differential toxicity towards transformed versus normal cells, TRAIL shows a potential cancer therapy agent; nevertheless, its safety must be further confirmed by ongoing clinical trials.

Regarding TRAIL, there remain numerous unanswered questions that will need further exploration. From my point of view, following belong to the most interesting:

- Multiplicity of TRAIL receptors. It seems probable that long-lived animals need fine tuned function of TRAIL that is provided by more complex TRAIL-R system; however the deeper background of TRAIL evolution remains unknown.
- Involvement of kinases and NF-κB in TRAIL pathways and. Today it is likely that NF-κB has mainly anti-apoptotic function in TRAIL signaling, yet roles of JNK and ERK are not clear at all.
- Role of post-translational modifications of individual components in TRAIL-induced pathways.
- Interaction with other apoptotic pathways. Evidence in given that signaling from DRs uses many common components; however complete scheme of crosstalk is unclear.
- Roles of controversial components of TRAIL signaling pathways such as caspase-10 and decoy rceptor-1, existence of PLAD in TRAIL receptors and forming of TRAIL-R heterotrimers, influence of FADD phosphorylation on TRAIL signaling.
- Biology of TRAIL signaling in stem cells and cancer stem cells. (This will be also scope of my future work, see below.)

9. Outlines of author's future experimental work

My experimental project for diploma thesis will be focused on characterization and regulation of TRAIL-induced apoptosis in small subpopulation of cancer cells, so called tumor initiating cells or cancer stem cells. These cells lately attracted significant attention and appear to be one of the reasons for fatal relapses in the current therapy of many tumors. These cells are apparently fairly resistant to apoptosis, and thus characterization of the induction and regulation of TRAIL signaling in cancer stem cells would be of an importance also in the regard to ongoing clinical tests with TRAIL-related therapeutics.

9.1 Cancer stem cells

Recent data from both hematologic malignancies and solid tumors suggested that there are only minor populations of cells in each malignancy that are capable of tumor initiation. For example, when mouse myeloma cells were obtained from mouse ascites, separated from normal haematopoietic cells and put in clonal *in vitro* colony-forming assays, only 1 in 10^4 to 1 in 10^2 cancer cells were able to form colonies (105). These tumor initiating cells have the functional properties of stem cell and thus are also named as tumor stem cells. They appear to be capable of asymmetric division and self renewal (Fig. 5), and represent only a minor faction among the bulk of more differentiated tumor cells. These observations may have profound implications for tumor biology research as well as for successful tumor therapy. Although currently used chemotherapy eliminates most of tumor cells, it apparently often due to their increased apoptosis resistance spares tumor stem cells and these are then a reason for fatal relapses of the disease. For example the ATP-binding cassette (ABC) drug transporter, which is also typical for normal stem cells, have been shown to render cancer stem cells the multidrug resistance (106). In context of these findings a theory of tumor initiating cells origin from normal stem cells has risen. Although normal stem cell can self-renew, they are generally quiesctent spending most of their time in G0 phase. Because stem cells can repair DNA as they self-renew, they have potential to accumulate mutations acquired after exposure to carcinogens. Alternative theory proposes that cancer stem cells arise from differentiated cells that acquire self-renewal capacity. It is possible that both are correct, yet it will need further investigation (105). Given the evidence that stem cells development is regulated by many signaling pathways that are associated with apoptosis and dysregulated in cancer (e.g., enforced expression of Bcl-2 (107)), it will be certainly exciting to find out, if/how the signaling from death receptors (and namely TRAIL-Rs) is influenced and if they could be potentially used for elimination of these tumor initiating cells. This will be also major part of my diploma project.

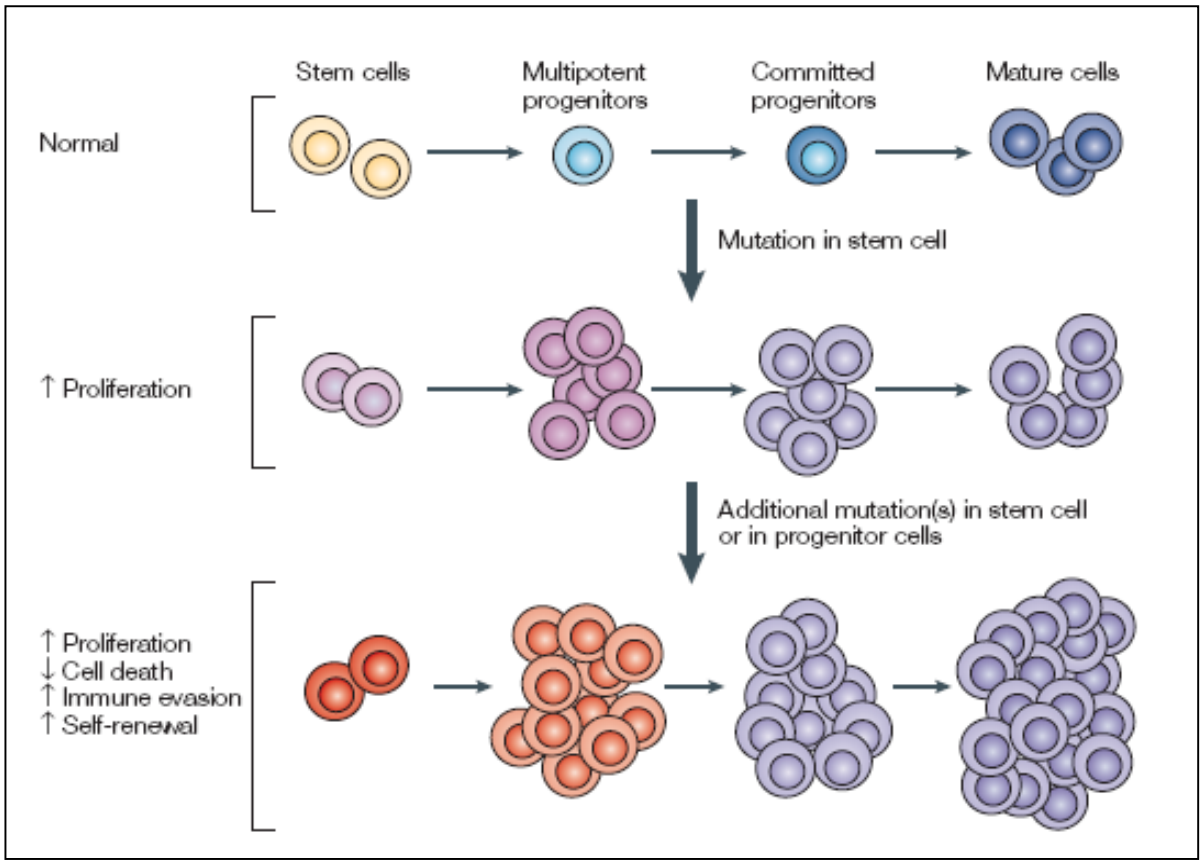

Figure 5 Cancer stem cells and tumour progression. Normal stem cells give rise to multipotent progenitors, committed progenitors and mature cells. Acquiring mutations leads to abberant proliferation and pre-malignant lesion formation. Further accumulation of mutations leads to decreased apoptosis evasion of the immune system and forming of tumor; ad ref. (101).

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