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TRAIL-Induced Apoptosis

Between Tumor Therapy and Immunopathology

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The death ligand members of the tumor necrosis factor (TNF) family are potent inducers of apoptosis in a variety of cell types. In particular, TNF-related apoptosis-inducing ligand (TRAIL) has recently received much scientific and commercial attention because of its potent tumor cell-killing activity while leaving normal untransformed cells mostly unaffected. Furthermore, TRAIL strongly synergizes with conventional chemotherapeutic drugs in inducing tumor cell apoptosis, making it a most promising candidate for future cancer therapy. Increasing evidence indicates, however, that TRAIL may also induce or modulate apoptosis in primary cells. A particular concern is the potential side effect of TRAIL-based tumor therapies in the liver. In this review we summarize some of the recent findings on the role of TRAIL in tumor cell and hepatocyte apoptosis.

Key words: **TRAIL; hepatocyte; apoptosis; tumor; liver injury; Bim; Jun kinase**

Introduction

To preserve proper physiological functions of multicellular organisms, the total number of cells must be tightly controlled. The absolute number of cells is not only controlled by cell division and proliferation but also by cell death. Apoptosis, or programmed cell death, plays an essential role in the elimination of injured or unwanted cells in many physiological and pathophysiological conditions. Excessive apoptosis has been associated with degenerative diseases, such as Alzheimer's disease, while a failure to complete apoptosis may result in cancer. Apoptosis induction is one of the major targets of modern cancer therapy. Consequently, a plethora of chemical and biological compounds have been developed that interfere with the metabolism and biochemistry of tumor cells and ultimately induce apoptosis. However, several tumor cells have developed various apoptosis resistance mechanisms, rendering many of these treatments ineffective. Thus, alternative approaches are desperately needed. The death-inducing members of the tumor necrosis factor (TNF) superfamily, that is, TNF-α, Fas (CD95) ligand, and TNF-related apoptosis-inducing ligand (TRAIL) represent interesting candidates for future anticancer therapy. In this review we will mainly focus on the apoptosis-regulating activities of TRAIL in tumor and primary cells.

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TRAIL Signaling Pathways

TRAIL was identified by two independent groups based on its sequence homology with Fas ligand and TNF-α.[1,2] TRAIL is a type II transmembrane protein in which the carboxyl terminus with the receptor-binding domain protrudes extracellularly. Similar to TNF-α and Fas ligand, transmembrane TRAIL can

be cleaved by metalloproteases to yield a soluble form, which maintains its biological activity.[3] Like all members of the TNF family, TRAIL also forms trimers. In addition, TRAIL activity is further regulated by a zinc ion bound to a cystein residue (Cys 230) at the trimer's interface.[4] Human TRAIL can bind to five different receptor molecules, TRAIL-R1, R2, R3, R4, and osteoprotegerin (OPG).[5] Two of these receptors, TRAIL-R1 and R2, contain a cytoplasmic so-called death domain capable of recruiting apoptosis signaling molecules and inducing cell death. In contrast, TRAIL-R3 and R4 do not contain functional death domains and may instead act as decoy receptors, likely by sequestering TRAIL and preventing activation of TRAIL-R1 and R2. However, direct nonapoptotic signaling via these receptors may also be possible and has not yet been fully excluded. Although OPG can bind TRAIL, its affinity to TRAIL is much weaker than that of TRAIL-R1–R4 and likely not relevant under physiological conditions. TRAIL receptors are ubiquitously expressed, indicating that most tissues and cell types are potential targets of TRAIL signaling.[6] TRAIL receptor-induced apoptosis in these tissues is likely tightly regulated, as TRAIL and TRAIL receptor expression in primary tissues is not associated with increased apoptosis. Interestingly for its potential use in cancer therapy and the subject of this review, primary cells appear to be largely insensitive to the apoptosis-inducing activity of TRAIL, while many tumor cells undergo apoptosis upon TRAIL receptor triggering.

The apoptosis signaling cascade initiated by the TRAIL receptors has been analyzed in detail, although new aspects may be discovered in the future. Similar to Fas and TNF-R1 signaling, TRAIL-R-induced apoptosis largely depends on recruitment of signaling molecules by homologous protein–protein interactions, forming the DISC (death-inducing signaling complex).[7] Activation of the receptors upon binding of the ligand induces conformational changes, which lead to the recruitment of the adaptor molecule FADD (Fas-associated death domain) via homologous death domain–death domain interactions. FADD, in turn, can recruit inactive caspase-8 via so-called death effector domain interactions. Once caspase-8 is activated at the DISC, it has two preferred substrates in the apoptosis pathways: pro-caspase-3 and Bid. Cleavage and subsequent autocatalytic activation of caspase-3 directly triggers a caspase cascade, ultimately leading to apoptosis, whereas cleavage of the pro-apoptotic Bcl-2 family member Bid links the receptor-mediated extrinsic pathway and the intrinsic pathway, which are discussed below.

TRAIL and Bcl-2 Family Members

In mammalian cells two major mechanisms have been described to control cell death. They are summarized in the so-called extrinsic and intrinsic pathways. The extrinsic pathway is activated by ligation of death receptors, whereas the intrinsic apoptosis pathway is initiated by signals that activate the mitochondrial apoptosis trail including growth factor withdrawal, cytotoxic and DNA-damaging agents, such as chemotherapeutic drugs. The intrinsic pathway is regulated by the interplay between pro- and anti-apoptotic members of the Bcl-2 family. Death-inducing signals activate so-called BH3-only (containing only Bcl-2-homology domain 3) proteins of the pro-apoptotic subgroup of the Bcl-2 family (e.g., Bim), which may than initiate the mitochondrial apoptosis pathway through inactivation of anti-apoptotic Bcl-2 homologues or activation of pro-apoptotic Bcl-2 members. While in many cell types these two principle signaling pathways may proceed completely independently, there is also considerable cross-talk between the extrinsic and intrinsic pathway. In type I cells, TRAIL-R-induced caspase-8 activation directly results in downstream caspase activation and apoptosis, whereas in type II cells caspase-8 activation is ineffective and

the signal must be amplified through caspase-8-mediated cleavage of the proapoptotic Bcl-2 homologue Bid and activation of the mitochondrial apoptosis pathway.[8] Many tumor cells are type II cells, and several studies have demonstrated that TRAIL strongly synergizes with conventional chemotherapeutic drugs in inducing tumor cell apoptosis, making it a most promising candidate for future cancer therapy. However, not only tumor cells but also hepatocytes are type II cells, requiring the activation of the mitochondrial pathway for apoptosis induction via trigger of the death receptor pathway. Thus, TRAIL receptor signaling may also synergize with the mitochondrial apoptosis pathway in primary cells, such as hepatocytes, and promote undesired side effects and tissue damage.

Nonapoptotic TRAIL Signaling

In addition to its proapoptotic function, TRAIL has been shown to initiate mitogenic and prosurvival signals that include activation of nuclear factor (NF)-κB, protein kinase B (PKB or Akt) and mitogen-activated protein (MAP) kinases.[9–12] Similar to TNF-α, TRAIL can activate NF-κB via the receptor-interacting protein (RIP), which is recruited by its death domain to the TRAIL–TRAILR complexes.[13] Upon TRAIL receptor triggering, RIP interacts with NF-κB essential modulator (NEMO/IKKγ), resulting in the recruitment of IκB kinases (IKKβ), and IKKβ in turn phosphorylates IκB, leading to its proteosomal degradation and activation of NF-κB.[14] Recent studies have suggested that inhibition of apoptosis via caspase inhibitors enhances the ability of TRAIL to stimulate the NF-κB pathway. For example, in TRAIL-resistant cells the predominant signal engaged by TRAIL–TRAIL receptor interaction is the activation of NF-κB, suggesting that resistance to apoptosis may favor TRAIL signaling pathways leading to NF-κB activation. Accordingly, it has been shown that TRAIL receptor activation results in NF-κB-dependent formation of cholangiocarcinoma metastases.[15] In addition, TRAIL-R1 and TRAIL-R2 have been shown to activate PKB/Akt and MAP kinases, in particular the extracellular signal-regulated kinase (ERK), JNK (c-Jun N-terminal kinase) and p38. Several reports have suggested that TRAIL-induced ERK activation results in apoptosis resistance. This was supported by the finding that ERK activation suppresses TRAIL-mediated apoptosis and inhibits the processing of caspase-8 and Bid.[12,16] In agreement with this finding was the fact that TRAIL-induced apoptosis is increased by inhibition of the ERK signaling pathway in human breast cancer cells.[17] On the other hand, a recent study demonstrated a proapoptotic role for TRAIL-mediated activation of ERK. Here, PG490, a diterpene triepoxide purified from the Chinese herb *Tripterygium wilfordii* sensitizes lung cancer cells to TRAIL-induced apoptosis. This sensitization effect can be blocked when ERK phosphorylation is inhibited.[18] Several controversial results have been published concerning the role of p38 and JNK activation in TRAIL-induced apoptosis. Earlier studies indicated that TRAIL-induced JNK activation in HeLa cells was not associated with cell apoptosis.[19] Similarly, Zhang *et al.* demonstrated that TRAIL-induced p38 activation in human colon cancer cell lines does not correlate with TRAIL-mediated cell death.[20] In contrast to these reports, we and others demonstrated recently that TRAIL can induce JNK phosphorylation with direct implications for caspase activation and apoptosis.[21,22]

TRAIL Sensitivity and Resistance

None of the apoptosis-inducing members of the TNF family has received as much attention in cancer research as TRAIL. The reason for this strong interest is the fact that recombinant TRAIL specifically induces apoptosis in a broad range of tumor cells while leaving normal nontransformed cells mostly unaffected. Furthermore, TRAIL strongly synergizes with

conventional chemotherapeutic drugs in inducing tumor cell apoptosis, making it a most promising candidate for future cancer therapy. While this selectivity for tumor cells is not absolute, clearly TRAIL is a more potent inducer of apoptosis in tumor cells than in primary cells. In marked contrast, both TNF-α and Fas ligand are known for a wide variety of proinflammatory or proapoptotic activities in normal untransformed immune and tissue cells. The reason for this differential sensitivity to TRAIL is only partially understood. Initially it was attributed to the different expression patterns of agonistic and antagonistic TRAIL receptors in normal and tumor cells. However, detailed analysis and the availability of specific antibodies revealed that there is no clear correlation between agonistic and antagonistic TRAIL receptor expression in resistant primary cells and sensitive tumor cells. In addition, agonistic TRAIL receptors (TRAIL-R1 and R2) were found to be widely expressed in both tumor and normal tissues.[6]

Thus, specific inhibitors of TRAIL receptor signaling rather than differential TRAIL receptor expression may determine TRAIL sensitivity or resistance. One of these candidates is cellular FLICE-like inhibitor protein (cFLIP). Several reports have indicated that overexpression of cFLIP results in resistance to TRAIL-induced apoptosis in different tumor cells.[23,24] For instance, Siegmund *et al.* demonstrated that selective inhibition of cFLIP expression with small-interfering RNA oligonucleotides is sufficient to sensitize tumor cells for TRAIL-induced apoptosis.[24] Whereas the caspase-8 homologue cFLIP may inhibit TRAIL-induced apoptosis right at the DISC, antiapoptotic Bcl-2 homologues can antagonize the amplification of the TRAIL receptor signal via the mitochondria. Furthermore, X-linked inhibitor of apoptosis protein (XIAP) has been shown to play a nonredundant role in TRAIL-mediated apoptosis in human cancer cells.[25,26] All these reports indicate that TRAIL sensitivity can be regulated at different levels depending on the cell type.

TRAIL as an Anticancer Agent

The fact that TRAIL induces apoptosis in a wide range of tumor cell lines has led to the proposal to use recombinant TRAIL as a specific anticancer agent. The potential of TRAIL therapy has been demonstrated in human tumor xenograft experiments in immunodeficient mice.[27] In these *in vivo* experiments TRAIL treatment substantially inhibited growth of a variety of human tumors, including breast and colon carcinomas, gliomas, and multiple myelomas. Critically, TRAIL treatment did not appear to have any side effects on normal tissue cells. While other death ligands (i.e., TNF-α and Fas ligand) can induce apoptosis in tumor cells, their systemic *in vivo* administration causes rapid death by inducing shock and/or liver toxicity.[28,29]

p53 is an important tumor suppressor molecule and induces cell cycle arrest and apoptosis. Most of the DNA-damaging chemotherapeutic drugs and radiation induce tumor cell apoptosis by triggering p53-mediated activation. The p53 gene is one of the most frequently mutated genes in tumor cells. Subsequently, p53-negative tumor cells are often resistant to conventional chemotherapy.[30] As TRAIL can induce apoptosis in many p53-negative tumor cells, this death trigger provides an attractive alternative to conventional p53-dependent tumor therapy.

The synergistic action of TRAIL and chemotherapy and radiotherapy is another potential bonus of TRAIL-based tumor therapy. Various *in vitro* and *in vivo* experiments have shown that combined treatment of tumor cells with TRAIL and conventional chemotherapeutics or irradiation strongly increases the sensitivity of tumor cells and leads to increased apoptosis and better eradication of the tumor. For example, TRAIL has been shown to synergize with γ-irradiation, 5-flucorouracil, cisplatin, etoposide, histone deacetylase inhibitors, CPT-11, and others and to cause increased cell death in a variety of tumor cells.[16,22,31–33] This synergy has various

reasons. As mentioned above, chemotherapy- and irradiation-induced DNA damage may activate p53, which in turn can induce an increased expression of TRAIL-R2 via p53 response elements in its promoter.[34] On the other hand, chemotherapy may also result in reduced expression of antiapoptotic molecules, such as cFLIP, with a direct effect on caspase-8 activation or Bcl-xL and XIAP, which in turn facilitate TRAIL-induced apoptosis via the mitochondrial pathway. For example, the proteosomal inhibitor bortezomib inhibits NF-κB activation and enhances TRAIL-induced apoptosis by reduction of cFLIP expression.[35,36] Similarly, the topoisomerase inhibitor CPT-11 can activate phosphotyrosine phosphatases and inactivate the signal transducer and activator of transcription (STAT)3/5, resulting in reduced Bcl-xL and XIAP expression and increased mitochondrial apoptosis.[37]

On the TRAIL side, it has been shown that TRAIL treatment can activate JNK, which has pro-apoptotic activities in a variety of tumor cells.[38] The molecular requirements for TRAIL receptor-induced JNK activation are somewhat controversial. While most reports agree that recruitment of FADD to the receptor is required for JNK activation, the involvement of caspase-8 is less clear and may be different in different cell types. For example, TRAIL treatment of caspase-8-deficient Jurkat cells or pretreatment of Jurkat or Hela cells with the pan-caspase inhibitor z-VAD does not lead to JNK activation, whereas, for example, in mesothelioma cells TRAIL-induced JNK activation is not blocked by z-VAD.[39] In contrast, recruitment of the death domain-containing kinase RIP1 and TRAF2 to the receptor complex seems to facilitate downstream JNK activation.[40,41] Interestingly, JNK activation likely plays a role in the synergistic induction of apoptosis by TRAIL and chemotherapeutics as overexpression of dominant negative JNK1 or pharmacological inhibition of JNK results in reduced apoptosis.[42] As both TRAIL and chemotherapeutics activate JNK, these experiments do not, however, reveal whether TRAIL-induced or chemotherapy-induced JNK activation is critical for this synergistic induction of cell death.

The relevant downstream targets of JNK for the death-inducing synergy between TRAIL and chemotherapy are also not well understood. Clearly, targets of JNK must somehow interfere with the apoptosis signaling pathway. The sensitizing effect of protein synthesis inhibition on TRAIL-induced apoptosis makes it unlikely that c-Jun/Fos (AP-1)-mediated gene expression may be relevant for this synergy. Of interest are recent findings that JNK can phosphorylate and activate the proapoptotic Bcl2 member Bim_{EL} and Bim_{L}.[21,43] Cai *et al.* demonstrated that p38 as well as JNK mediate apoptosis through phosphorylation of Bim_{EL} at S65.[44]

Role of TRAIL in Liver Damage

The proposed use of TRAIL in tumor therapy largely depends on its potency of apoptosis induction in tumor cells and on its potential side effects in normal tissue cells, in particular in the liver. Several findings reported in the literature point out that TRAIL-mediated killing of hepatocytes may represent one of the potential risks of TRAIL-based tumor therapy. Initial experiments using the transfer of human tumor cells into immune-deficient mice revealed that treatment with TRAIL exhibited tumoricidal activity without any obvious adverse side effects on the liver.[27] In agreement with these results, Takeda *et al.* reported that TRAIL is constitutively expressed on murine natural killer (NK) cells in the liver. Interestingly, liver NK cells appear to play a critical role in the control of liver metastases via TRAIL-based mechanisms but seem to be quite well tolerated by normal liver tissue.[45] Freshly isolated liver NK cells express relatively high levels of TRAIL at the cell surface, which seems to contribute to spontaneous NK cell-mediated cytotoxicity against TRAIL-sensitive tumor cells *in vitro*. Along these lines it was found that neutralization of TRAIL *in vivo* using anti-TRAIL

antibodies leads to increased liver metastases of transplanted TRAIL-sensitive tumor cell lines.[27] Similarly, TRAIL-deficient mice show a reduced capacity to eliminate tumor metastases in the liver.[46] Thus, these findings support a proposed role for TRAIL as a tumor-specific treatment in anticancer therapy with minimal side effects in normal tissue.

This conclusion is, however, also contrasted by several reports demonstrating TRAIL-mediated cell death induction in murine and human hepatocytes. For example, it was found that TRAIL-based hepatocyte killing is involved in the pathogenesis of viral hepatitis.[47] Likely, the viral infection of hepatocytes may change them to a more tumor cell-related phenotype and thereby increase their susceptibility to TRAIL treatment. On the other hand, however, it was also reported that freshly isolated human hepatocytes can be killed by recombinant TRAIL *in vitro*.[48] While this unexpected sensitivity of human hepatocytes could be partially attributed to the stress that hepatocytes may be exposed to during isolation and *ex vivo* culture or the use of different TRAIL preparations with different death-inducing activities, these observations also point out that TRAIL can induce hepatocyte apoptosis and liver damage under certain circumstances. Several lines of evidence indicate that stress or inflammation may render hepatocytes more sensitive to TRAIL-induced apoptosis. In accordance with this notion, we observed that isolated murine as well as human hepatocytes from healthy donors are not sensitive to TRAIL-induced apoptosis *in vitro*. In marked contrast, however, TRAIL-mediated hepatocyte apoptosis seems to contribute to experimental hepatitis after injection of the lectin concanavalin A, as TRAIL-deficient mice were significantly protected (unpublished results and Ref. 49). While this differential sensitivity of hepatocytes *in vitro* and *in vivo* is confusing, these findings also suggest that *in vivo* TRAIL signaling may cooperate with other death signals in promoting hepatocyte death. Of interest in this regard is the fact that not only TRAIL but also other death ligands, such as TNF-α and FasL, have been associated with lectin-induced liver damage and hepatitis. It is indeed well established that FasL is expressed in activated liver T cells and NKT cells contribute to liver damage in experimental hepatitis.[50] These results together with the fact that injection of recombinant TRAIL does not cause any signs of hepatitis suggest that TRAIL alone is not sufficient to initiate apoptosis in healthy hepatocytes but either requires the contribution of other death triggers, such as FasL, or that TRAIL may actually be a modulator of hepatocyte apoptosis induced by other triggers. In support of this notion, we found that recombinant TRAIL alone does not induce apoptosis in isolated murine hepatocytes; however, TRAIL strongly synergized with FasL in promoting hepatocyte cell death. More importantly, TRAIL-deficient mice were significantly more resistant to liver damage and associated death in response to injection of an agonistic anti-Fas antibody than control animals.[21]

Modulation of the Mitochondrial Apoptosis Pathway by TRAIL

These findings indicate that TRAIL does not induce hepatocyte apoptosis but can enhance the Fas signaling apoptosis pathway. How can this be explained? The Fas receptor signaling pathway can either lead to direct caspase activation (type I cells) or may require the amplification of the signal through caspase-8-mediated cleavage of Bid and activation of the mitochondrial pathway (type II cells). Hepatocytes are the best established example for primary type II cells. Thus, transgenic overexpression of Bcl-2 or deficiency in Bid renders mice particularly resistant to anti-Fas-induced hepatitis.[51,52] This amplification loop via signals activating the mitochondrial pathway is not specific for FasL but has been shown to play an essential role in TNF-α-induced liver injury. For example, Zhao *et al.* demonstrated that Bid-deficient mice were also partially protected from lipopolysaccharide/

D-galactosamine-induced TNF-α-dependent liver injury.[53] Our results suggest that TRAIL contributes to the amplification of the mitochondrial apoptosis signaling pathway in hepatocyte apoptosis. While TRAIL alone fails to induce apoptosis, it still triggers the activation of MAP kinases, in particular activation of JNK. We and others have found that JNK plays a critical role in various forms of hepatocyte apoptosis and liver damage.[21,54] For example, it was shown that free-fatty acids induce hepatocyte lipo-apoptosis in a TRAIL- and JNK-dependent manner.[54] Searching for relevant JNK targets, we identified the BH3-only molecule Bim as a critical JNK-regulated response modifier in the TRAIL-initiated enhancement of hepatocyte apoptosis. JNK phosphorylates Bim_{EL} and Bim_{L} *in vitro* and *in vivo*,[43,55] and in contrast to ERK1/2-mediated phosphorylation and degradation by the proteosome, this leads to a dissociation of Bim from the cytoskeleton and to its activation. In agreement with this proposed pathway, we observed that inhibition of JNK and absence of TRAIL or Bim renders mice largely resistant to anti-Fas-induced liver damage. We thus propose that TRAIL-initiated activation of Bim represents a second mitochondrial amplification loop, which further amplifies caspase-8- and Bid-initiated apoptosis signaling in hepatocytes after Fas receptor ligation. TRAIL-mediated enhancement of the mitochondrial pathway is also likely not restricted to Fas signaling in type II cells, such as hepatocytes, but may further extend to other triggers of the mitochondrial apoptosis pathway.[56]

The question arises why TRAIL-induced signaling pathways in the liver are regulated in such a complex manner. As mentioned above, liver-homing NK and NKT cells are a rich source of TRAIL, and TRAIL-mediated cytotoxicity also seems to play an important role in the immune cell-mediated control of liver metastases.[45] Clearly, if hepatocytes are intrinsically sensitive to TRAIL-induced apoptosis, the constitutive expression of TRAIL in liver NK and NKT cells would be a constant threat and likely lead to chronic liver destruction. Thus, it seems important that under normal conditions hepatocytes are insensitive to TRAIL-induced apoptosis. The proposed amplification of the mitochondrial apoptosis pathway by the TRAIL–JNK–Bim axis may, on the other hand, ensure appropriate hepatocyte apoptosis in response to other triggers and under certain circumstances (e.g., when hepatocyte apoptosis is needed for the protection of the host). Of particular interest in this regard is the fact that viral infection renders hepatocytes sensitive to TRAIL-induced apoptosis. Thus, TRAIL may represent a safeguard for the liver, allowing the elimination of infected or transformed cells while sparing normal liver cells required for the vital functions of this important organ.

Conflicts of Interest

The authors declare no conflicts of interest.

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