Role of IG20 Splice Variants in TRAIL Resistance
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Abstract
Tumor necrosis factor receptor–related apoptosis-inducing ligand (TRAIL) can induce apoptosis primarily in cancer cells with little or no effect on normal cells; therefore, it has the potential for use in cancer therapy. TRAIL binding to death receptors DR4 and DR5 triggers the death-inducing signal complex formation and activation of procaspase-8, which in turn activates caspase-3, leading to cell death. Like FasL, TRAIL can trigger type 1 (caspase-8 → caspase-3) or type 2 (caspase-8 → Bid cleavage → caspase-9 → caspase-3) apoptotic pathways depending on the cell type. Some cancers are resistant to TRAIL treatment because most molecules in the TRAIL signaling pathway, including FLIPs and IAPs, can contribute to resistance. In addition, we have identified an essential role for splice variants of the IG20 gene in TRAIL resistance.

Background
Given the role of tumor necrosis factor receptor–related apoptosis-inducing ligand (TRAIL) in apoptosis and its expression on immune cells, it is likely to contribute significantly to immune surveillance of virus-infected and cancer cells. TRAIL has the ability to kill tumor cells, with little or no effect on most normal cells. Systemic administration of TRAIL can cause tumor shrinkage/elimination, without causing significant side effects (reviewed in ref. 1). TRAIL in combination with chemotherapy and UV radiation can be more effective. In contrast to FasL or tumor necrosis factor α–based treatments that are associated with fulminant hepatitis and systemic inflammation, respectively, TRAIL administration seems to lack these side effects; therefore, TRAIL is an attractive candidate for cancer therapy (1–6). TRAIL is expressed as a trimeric type II transmembrane protein on mainly natural killer and natural killer–T cells. TRAIL can bind to five distinct receptors—death receptor 4 (DR4 or TRAILR-1), death receptor 5 (DR5 or TRAILR-2), decoy receptor 1 (DcR1, TRAILR-3, LIT or TRID), decoy receptor 2 (DcR2, TRAILR-4, TRUNDD), and osteoprotegerin. Among these, only DR4 and DR5 contain death domains and are able to mediate the death signal. DcR1 and DcR2 engage TRAIL, but they fail to signal. Osteoprotegerin is a soluble receptor and its affinity for TRAIL is weak.

TRAIL-mediated apoptosis signaling in cancer cells is similar to that seen with Fas (CD95). DR4 and DR5, through their cytoplasmic death domains, bind to the FADD death domain, which in turn interacts with procaspase-8 (or caspase-10) through its death effector domain. TRAIL binding causes increased recruitment of proteins to this death-inducing signaling complex (DISC) leading to proximity-induced caspase-8 activation. This causes subsequent caspase-3 (or caspase-7) activation, resulting in degradation of inhibitor of caspase-activated DNase. Proteolysis of inhibitor of caspase-activated DNAase releases CAD that cleaves the genomic DNA (refs. 7, 8; Fig. 1).

In addition to the type 1 pathway, TRAIL-mediated apoptosis can also recruit the intrinsic or type 2 pathway under conditions in which the caspase-8 levels are limiting and the proapoptotic molecule Bid is expressed. The intrinsic cell death pathway is regulated through a variety of Bcl2 family members that act primarily in mitochondria and endoplasmic reticulum. The antiapoptotic members of the Bcl2 family, such as Bcl2, Bcl-xL, and Mc1l, contain four Bcl2 homology (BH1-BH4) domains, whereas proapoptotic members such as Bax and Bak lack BH4 domain. In healthy cells, Bcl2 and Bcl-xL neutralize the activity of Bax/Bak. The equilibrium between the antiapoptotic and proapoptotic Bcl2 family members is regulated by the mobilization of a third class of proapoptotic Bcl2 family members such as Bid, Bad, Puma, and Noxa. In a type 2 pathway, the activated caspase-8 cleaves Bid to form tBid whose BH3 domain is now exposed, allowing it to interact with Bcl2/Bcl-xL and trigger intrinsic cell death pathway (9, 10). A disruption in the equilibrium results in oligomerization of Bax/Bak at mitochondrial outer membrane, leading to loss of mitochondrial integrity and leakage of cytochrome c into cytosol. Cytochrome c then interacts with Apaf1 and procaspase-9 and facilitates oligomerization and activation of caspase-9 that results in caspase-3 activation (ref. 11; see Fig. 1).

Cellular FLICE Inhibitory Proteins in TRAIL Resistance
Apoptotic pathways are kept in check by cell proliferation and survival signaling. For instance, c-FLIP is an inhibitor of death receptor signaling as it competes with caspase-8 to bind to FADD (12). Structurally, c-FLIP closely resembles caspase-8 but lacks the caspase-8 enzymatic activity due to substitution of a key cysteine with aspartate in the active site. Some of the
c-FLIP isoforms [e.g., c-FLIPL (p55), c-FLIPS (p26), c-FLIPR (p24), and p22] also activate the prosurvival transcription factor nuclear factor-κB (NF-κB). The p22 form can associate with NEMO, and be a part of the IKK complex causing NF-κB activation. In addition, c-FLIPL is an NF-κB–responsive gene and, on expression, it can inhibit Fas as well as TRAIL-induced apoptosis (refs. 12–16; Fig. 1). The death effector domain in the FLIPs competes with the DED of caspase-8 for binding to FADD in the DISC, thereby inhibiting caspase-8 activation. Compared with FLIPL, FLIPS that lacks even the inactive protease domain seems to be much more effective in caspase-8 inhibition.

Evidence for the altered expression of this pathway has been reported in multiple types of cancer. TRAIL-resistant endometrial carcinomas express relatively high levels of c-FLIP and its down-modulation using either actinomycin D or specific small interfering RNA renders the carcinoma highly sensitive to TRAIL killing. Herpes virus–induced Kaposi’s sarcoma shows elevated levels of FLIP due to the production of V-FLIP. This can increase the basal levels of NF-κB activity, leading to enhanced expression of prosurvival genes that contribute to higher incidence of cancers. Elevated c-FLIP levels have been linked to TRAIL resistance in human melanomas; B-cell lymphomas; Reed-Steinberg’s cells of Hodgkin’s lymphoma; and carcinomas of...
the prostrate, the stomach, and the urinary bladder. Similarly, elevation in c-FLIP levels along with survivin is often observed in the highly fatal form of glioblastoma multiforme (17–27). A related member PED that targets caspase-8 and is highly expressed in B-cell chronic lymphocytic leukemia may confer TRAIL resistance (28). Specific NF-κB inhibitors can be administered to down-modulate not only c-FLIPs but also IAPs to sensitize TRAIL resistance carcinomas to TRAIL-mediated apoptosis (ref. 29; Fig. 2).

**Inhibitors of Apoptosis in TRAIL Resistance**

Inhibitors of apoptosis (IAP) proteins block apoptosis by binding either to effector caspase-3 and caspase-7 and/or to initiator caspase-9. They have one or three tandem baculoviral inhibitory repeat (BIR) domains. To date, six IAPs have been identified—cIAP1, cIAP2, X-IAP (X linked), NIAP (neuronal), Survivin, and BRUCE (Bir repeat containing ubiquitin conjugating enzyme), of which XIAP is the most potent. During intrinsic apoptosis, in addition to cytochrome c, Smac/Diablo is leaked into the cytoplasm wherein they interact with the BIR domains of IAPs and neutralize them. In type II cells, where caspase-8 activity is limiting, reduced or increased levels of Smac/Diablo or IAPs, respectively, can confer TRAIL resistance (reviewed in ref. 30). Many human cancers harbor high levels of IAPs and a majority of pancreatic carcinoma cell lines are TRAIL resistant due to poor release of Smac/Diablo from mitochondria (31, 32). Down-modulation of XIAP in NSCLC xenografts renders them highly susceptible to TRAIL treatment in mouse models (33–35). Although inhibition of IAPs with Smac peptides or with phenytoin may not be sufficient, it is very likely that in combination with TRAIL, it will significantly increase the efficacy of the treatment of cancers that are refractory to TRAIL treatment due to elevated levels of IAPs (refs. 36, 37; Fig. 2).

**Bcl2 Family Members in TRAIL Resistance**

The Bcl2 family members can contribute to TRAIL resistance that can be reversed with BH3 mimetic Nona peptides (38, 39). Surprisingly, in some prostate and colon cancer cells, TRAIL induces resistance through increased expression of the anti-apoptotic protein Bcl-xL, presumably due to activation of NF-κB (40). Mcl-1, an antiapoptotic member of the Bcl2 family, is highly expressed in multiple myeloma, hepatocellular carcinoma, and liver metastasis of colorectal carcinoma (41, 42) and confers resistance to various therapies including TRAIL. In pancreatic ductal carcinoma cells, expression of Bcl-xL not only confers resistance to TRAIL but also unravels the ability of TRAIL to activate the NF-κB–mediated prosurvival pathway and enhance metastasis. This was elegantly shown by orthotopic transplantation of ductal carcinoma cells into severe...
combined immunodeficient mouse pancreas. Only three TRAIL treatments in these mice were enough to trigger metastasis of the pancreatic cancer to liver, spleen, and peritoneum without any apparent effect on the growth of the primary tumor (43). A similar observation has been described in another study using cholangiocarcinoma cells. TRAIL treatment promoted tumor cell migration, and silencing of NF-κB had no discernible effect. In these cells, the TRAIL resistance was found to be caused by Mcl-1 and, unlike Bcl-xL, it is not regulated by NF-κB (44, 45). Therefore, a careful evaluation of the molecular nature of cancer is necessary before embarking on TRAIL treatment for cancers. The BH3 mimetic ABT737, which is a potent inhibitor of both Bcl2 and Bcl-xL, and others are currently in clinical trials and by themselves seem to be highly effective. They may not, however, overcome the resistance offered by a related BCL2 family member Mcl1 as seen in Fig. 2 (44).

**IG20 Isoforms and their Role in TRAIL-Induced Signaling**

The IG20 splice variants play important roles in cell survival, proliferation, and apoptosis, and vesicular trafficking. The IG20 gene can encode at least four different splice variants, namely IG20pa, MADD/DENN, IG20-SV2, and DENN-SV (46–50). IG20 isoforms play significant roles in cancer cell proliferation, survival, and death (50–56). Most cancer cells constitutively express MADD and DENN-SV with varying levels to no expression of IG20pa and IG20-SV2 (50). Earlier studies, using antisense oligodeoxynucleotides, showed that knockdown of all IG20-SVs can result in spontaneous apoptosis of cancer cells but not normal cells in vitro as well as in vivo (55–57). Although an indispensable role for IG20 in cancer cell survival was shown, these studies failed to delineate the relative importance of different IG20-SVs.

In our recent studies using short hairpin RNAs that specifically target either exon 15, which is expressed in all isoforms of IG20 (Mid), or exons 13L or 16, which are differentially expressed in IG20-SVs, we were able to selectively knock down either all or combinations of IG20-SVs in HeLa and PA-1 cells to determine their role in cell survival. The ovarian carcinoma PA1 cells are resistant to TRAIL-induced apoptosis and express only MADD and DENN-SV, indicating that either or both of these isoforms are indispensable for cell survival and/or TRAIL resistance. Knockdown of MADD, and not DENN-SV, enhanced spontaneous as well as TRAIL-induced apoptosis through caspase-8 activation. Further, endogenous MADD could inhibit caspase-8 activation, at the DISC, by sequestering the DR4/DR5 through direct interactions. MADD per se does not interact with caspase-8 or FADD nor does it affect their interactions with death receptors. In contrast, overexpression of IG20pa rendered even PA1 cells highly susceptible to TRAIL through enhanced recruitment of DISC and activation of caspase-8. Knockdown of endogenous IG20pa has no discernible effect on spontaneous or TRAIL-induced apoptosis, however. Although loss of DENN-SV has no apparent effect on apoptosis, expression of DENN-SV can lead to increased resistance to TRAIL, most likely through enhanced production of prosurvival factors through NF-κB activation (refs. 50, 52–54, 58, 59; Fig. 1).

Taken together, these studies show a prosurvival role for MADD, and expression of MADD especially in the absence of IG20pa seems to confer resistance to TRAIL-induced apoptosis. IG20pa, when expressed, behaves like a dominant negative MADD. It is therefore very likely that the constitutively expressed isoforms, MADD and DENN-SV, contribute to TRAIL resistance by inhibiting caspase-8 and activating NF-κB, respectively. Our results clearly show that knockdown of all IG20 splice variants or the MADD variant using a lentiviral vector that can express either mid or 13L small interfering RNA, respectively, not only induces significant apoptosis but also synergizes with TRAIL treatment (Fig. 2).

**Clinical-Translational Advances**

TRAIL seems to be an excellent choice of treatment for a variety of cancers. There is a distinct possibility, however, that the treatment itself can quickly induce resistance and promote metastasis through activation of NF-κB. In addition, intrinsic TRAIL resistance seems to occur more frequently than what was originally anticipated, and the signaling components in the TRAIL-mediated cell death pathway, such as FLIP and IAP, seem to play a significant role. Loss-of-function mutations in TRAIL receptors, overexpression of decoy receptors, and loss of caspase-8 expression due to gene methylation can also contribute to TRAIL resistance (60–67). Our recent studies with various splice variants of IG20 unraveled yet another level of regulation of TRAIL-induced cell death. Constitutive expression of the MADD and DENN-SV isoforms, especially in the absence of IG20pa and IG20–SV2, seems to confer TRAIL resistance. As shown in the figure, MADD seems to regulate the very proximal event in TRAIL signaling. It is possible that MADD can sequester DR4/DR5 receptors and thus negatively regulate TRAIL-induced apoptosis in addition to its inhibitory effect on endogenous caspase-8 activation at the DISC. Therefore, to maximize the efficacy of TRAIL, it is critical that we rapidly and reliably identify the potential TRAIL resistance mediators. A combinatorial approach that will target the key contributors to TRAIL resistance and includes TRAIL is likely to be a more effective therapeutic approach to cure cancer. A single combination is unlikely to be the cure all and thus a cancer-specific combinatorial therapy based on the molecular expression pattern of TRAIL resistance markers might be more effective (Fig. 2).

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