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TNF-related apoptosis-inducing ligand (TRAIL): A new path to anti-cancer therapies

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Abstract

Since its discovery in 1995, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor super family, has been under intense focus because of its remarkable ability to induce apoptosis in malignant human cells while leaving normal cells unscathed. Consequently, activation of the apoptotic signaling pathway from the death-inducing TRAIL receptors provides an attractive, biologically-targeted approach to cancer therapy. A great deal of research has focused on deciphering the TRAIL receptor signaling cascade and intracellular regulation of this pathway, as many human tumor cells possess mechanisms of resistance to TRAIL-induced apoptosis. This review focuses on the currently state of knowledge regarding TRAIL signaling and resistance, the preclinical development of therapies targeted at TRAIL receptors and modulators of the pathway, and the results of clinical trials for cancer treatment that have emerged from this base of knowledge. TRAIL-based approaches to cancer therapy vary from systemic administration of recombinant, soluble TRAIL protein with or without the combination of traditional chemotherapy, radiation or novel anticancer agents to agonistic monoclonal antibodies directed against functional TRAIL receptors to TRAIL gene transfer therapy. A better understanding of TRAIL resistance mechanisms may allow for the development of more effective therapies that exploit this cell-mediated pathway to apoptosis.

Keywords

Tumor necrosis factor-related apoptosis-inducing ligand; cancer immunotherapy; apoptosis; TRAIL receptor

1. Introduction

Despite continuous efforts to improve conventional cancer treatments, the long-term outcome for many patients treated with surgery, chemotherapy, and/or radiotherapy remains suboptimal. Furthermore, the lack of specificity of these approaches may cause significant toxicity. Cells damaged by chemotherapy or radiation are generally eliminated through apoptosis, a controlled

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biologic process resulting in cell death. Cancer is probably the most intensely investigated disease associated with defects in the apoptotic cell death process. Tumor cells can become resistant to chemotherapy by developing mechanisms to inhibit the apoptotic signaling process. Tumor cells from a wide array of human malignancies have demonstrated a decreased ability to undergo apoptosis, and a great deal of time and money are therefore being spent on the development of therapeutic agents targeting the apoptosis pathway within these cells.

Apoptosis can be initiated by two distinct, but interconnected, molecular signaling pathways. The first is the intrinsic pathway, which typically responds to severe DNA damage, hypoxia, or other cell stresses (such as that caused by chemotherapy or ionizing radiation). The intrinsic pathway is largely mediated and controlled by interactions of pro-apoptotic and anti-apoptotic members of the B-cell leukemia/lymphoma 2 (Bcl-2) protein family that can cause release of apoptosis-inducing factors from mitochondria (Wei et al., 2001).

The second is the extrinsic pathway, which induces apoptosis through an active, instructive process mediated by cell surface death receptors that transmit apoptotic signals when bound by their cognate death ligands and is of particular interest within cancer research. These death receptors are members of the tumor necrosis factor (TNF) receptor superfamily, and are characterized by cysteine-rich extracellular domains (Armitage, 1994; Smith et al., 1994). Additionally, all the death receptors contain a homologous cytoplasmic sequence called the death domain that serves as the recognition point for the apoptotic machinery (Itoh and Nagata, 1993; Smith et al., 1994; Tartaglia et al., 1993). The ligands for the death receptors belong to the TNF family of cytokines, a group of molecules that influence a variety of immunological functions. TNF and Fas ligand (FasL) are two of the most studied death ligands that induce apoptosis in many physiological events, such as autoimmunity, activation-induced cell death, immune privilege, and evasion of tumors from the immune system (Alderson et al., 1995; Cerami and Beutler, 1988; Griffith et al., 1995; Hahne et al., 1996; Zheng et al., 1995).

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is another member of the TNF superfamily that has emerged since its discovery in 1995 as a prominent biologically-targeted anti-tumor protein because of its remarkable ability to induce apoptosis in a variety of human cancer cell lines while leaving normal cells unaffected (Walczak et al., 1999). With this in mind, we will review here TRAIL structure and function, TRAIL receptor signaling, and mechanisms that confer TRAIL resistance, as well as highlight some of the preclinical and clinical investigations into the therapeutic utilization of TRAIL in the treatment of cancer.

2. Structure and Function of TRAIL

While searching for molecules containing a conserved sequence present in many TNF family members, Wiley *et al.* identified an expressed sequence tag that was then used to clone the full length TRAIL cDNA (Wiley et al., 1995b). A report by Pitti et al. (Pitti et al., 1996), published several months later, described the same protein – but was called Apo-2 ligand. Comparison of the extracellular domain of TRAIL found it is most homologous to Fas ligand (28% amino acid identity), but it also has significant identity to TNF (23%), lymphotoxin- α (23%), and lymphotoxin- β (22%). Whereas the homology of TRAIL to other TNF family members may be considered low, analysis of the crystal structure of monomeric TRAIL found it to be very similar to that of TNF and CD40 ligand (Cha et al., 1999). TRAIL monomers are made up of two antiparallel β -pleated sheets that form a β sandwich core framework, and the monomers are able to interact in a head-to-tail fashion to form a bell-shaped trimer (Cha et al., 1999). This oligomerization greatly enhances TRAIL activity as studies with recombinant soluble TRAIL found that multimeric, or crosslinked, forms possess more significant biological activity than monomeric versions of TRAIL (Wiley et al., 1995b). Interestingly, concerns about the toxic potential of TRAIL were raised by the fact that certain recombinant forms of soluble TRAIL

induced apoptosis in human hepatocytes *in vitro* (Jo et al., 2000). A TRAIL monomer of native sequence contains a single cysteine, Cys-230, and the cysteines from three monomers are in close proximity to one another in trimeric TRAIL, permitting Zn^{2+} chelation (Cha et al., 1999). By comparison, the poly-His tagged recombinant TRAIL version that demonstrated hepatocyte toxicity *in vitro* had a low Zn^{2+} content and adopted an aberrant 3-D structure compared to native TRAIL (Lawrence et al., 2001). Thus, it was concluded that the hepatotoxicity was an *in vitro* observation entirely dependent upon the form of TRAIL used, and the use of untagged TRAIL (a.k.a. Apo2L/TRAIL.0 (Lawrence et al., 2001)), which resembles native TRAIL, in a therapeutic setting should not be toxic.

Early investigation of TRAIL function identified two unique characteristics that were not observed for other TNF family death inducers (i.e. TNF and FasL). First, TRAIL preferentially induces apoptosis in tumorigenic or transformed cells, but not normal cells or tissues (Wiley et al., 1995b). Cells undergoing TRAIL-induced death exhibit many of the hallmarks of apoptosis, including DNA fragmentation, expression of pro-phagocytic signals (i.e. phosphatidylserine) on the cell membrane, and cleavage of multiple intracellular proteins by caspases (Griffith et al., 1998; Pitti et al., 1996; Wiley et al., 1995a). Second, TRAIL messenger RNA is expressed in a wide range of tissues, including peripheral blood lymphocytes, spleen, thymus, prostate, ovary, small intestine, colon and placenta; in contrast, the expression of other TNF family members is tightly regulated and often only transient (Wiley et al., 1995b). Within the immune system, TRAIL can be expressed by human T cells after CD3 crosslinking and type I interferon stimulation - perhaps contributing to the activation-induced cell death of T cells in the natural setting (Kayagaki et al., 1999). In addition, human natural killer cells, B cells, monocytes, and dendritic cells express membrane-bound TRAIL following cytokine stimulation (especially type I and II interferon), transforming them into potent tumor cell killers (Fanger et al., 1999; Griffith et al., 1999; Kemp et al., 2004; Zamai et al., 1998). Our group was also one of several to demonstrate that human polymorphonuclear neutrophils contain intracellular stores of TRAIL (Cassatella et al., 2006; Kemp et al., 2005; Koga et al., 2004; Ludwig et al., 2004; Tecchio et al., 2004) that can be released in a functional soluble form after appropriate stimulation (Kemp et al., 2005; Simons et al., 2008; Simons et al., 2007).

TRAIL has been most extensively studied as an inducer of tumor cell apoptosis, and appears to play a prominent role in normal tumor immunosurveillance as TRAIL knockout mice support tumor growth at a higher rate compared to normal mice and are more susceptible to tumor metastasis (Cretney et al., 2002). Additional studies have shown that the mouse TRAIL receptor may act as a negative regulator of innate immunity (Diehl et al., 2004). The widespread expression of TRAIL in normal tissues suggests a number of potential physiologic roles other than induction of apoptosis in neoplastic cells. Unfortunately, it has become clear that many primary tumor cells are resistant to TRAIL-induced apoptosis as the result of a variety of molecular alterations (Dyer et al., 2007).

3. TRAIL Receptor Signaling

A number of reports published in the late 1990's identified multiple receptors for TRAIL. In humans, TRAIL binds to four known membrane-bound receptors, Death Receptor 4/TRAIL receptor-1 (Pan et al., 1997b), Death Receptor 5/TRAIL receptor-2 (Pan et al., 1997a; Walczak et al., 1997), TRAIL receptor without an intracellular domain/Decoy Receptor 1/TRAIL receptor-3 (Degli-Esposti et al., 1997b; Pan et al., 1997a; Sheridan et al., 1997), and TRAIL receptor-4/Decoy Receptor 2/TRAIL receptor with a truncated death domain (Degli-Esposti et al., 1997a; Marsters et al., 1997; Pan et al., 1998) (hereafter referred to as TRAIL receptor-1, TRAIL receptor-2, TRAIL receptor-3, and TRAIL receptor-4, respectively, Fig. 1). The two "decoy" receptors TRAIL receptor-3 and TRAIL receptor-4 bind TRAIL, but do not possess an intracellular domain or contain a truncated intracellular death domain, respectively.

Therefore, TRAIL receptor-3 and TRAIL receptor-4 do not signal for apoptosis, and have been proposed to be competitive inhibitors to regulate TRAIL-induced apoptosis (Sheridan et al., 1997). By comparison, TRAIL receptor-1 and TRAIL receptor-2 contain a functional cytoplasmic death domain capable of activating the extrinsic pathway for apoptosis after TRAIL-induced receptor trimerization (Hymowitz et al., 1999). A multiprotein structure called the death-inducing signaling complex (DISC), which includes Fas-associated death domain protein (FADD), procaspase 8, and possibly cellular FADD-like IL-1 β -converting enzyme inhibitory protein (cFLIP), is recruited to and associates with trimerized TRAIL receptor-1 or TRAIL receptor-2 (Kischkel et al., 2000; Sprick et al., 2000). Procaspase 8 is autocatalytically cleaved and activated at the DISC, and can then cleave multiple intracellular substrates (such as the downstream effector caspases 3, 6 and 7) (Kischkel et al., 2000). Active caspase 8 can also cleave the pro-apoptotic Bcl-2 protein Bid, thus engaging the intrinsic pathway (Li et al., 1998). In some cells, activated caspase 8 is sufficient to trigger apoptosis (type I cells), while other cells require activation of the intrinsic pathway to amplify the apoptotic signal (type II cells) (Barnhart et al., 2003). Type II cells also require inactivation of intracellular apoptosis inhibitors, such as X-linked inhibitor of apoptosis protein (XIAP), which directly inhibit effector caspase activity. Once the intrinsic pathway is triggered, the integrity of the mitochondrial membrane is lost – leading to second mitochondria-derived activator of caspases/direct inhibitor of apoptosis protein binding protein with low isoelectric point (Smac/DIABLO) release, which acts as a natural inhibitor of XIAP (Du et al., 2000; Verhagen et al., 2000). In addition, cytochrome c escapes from the mitochondria, allowing it to bind to apoptotic peptidase activating factor 1 and caspase 9 to form the apoptosome (Li et al., 1997; Schulze-Osthoff et al., 1998). Together, inhibitor of apoptosis protein inhibition and apoptosome activation dramatically accelerate the apoptotic disassembly of the dying cell. A schematic of the TRAIL receptor signaling pathway is depicted in Fig. 2.

TRAIL can also bind to a soluble receptor, osteoprotegerin (Emery et al., 1998), which functions as a decoy receptor for Receptor Activator of NF- κ B Ligand (RANKL). RANKL activates NF- κ B through its membrane-bound receptor, Receptor Activator of NF- κ B, leading to osteoclast-mediated bone resorption. (Lacey et al., 1998) It has been proposed that TRAIL may play a role in bone homeostasis; however, TRAIL knockout mice demonstrate a normal skeletal phenotype.

4. Mechanisms of TRAIL resistance

While many reports have proven TRAIL to be a potent inducer of apoptosis in a wide variety of human and mouse tumor cell lines, many primary tumors are resistant to TRAIL-induced apoptosis (Dyer et al., 2007) and several mechanisms underlying TRAIL sensitivity/resistance have been proposed. TRAIL messenger RNA is constitutively expressed in a wide variety of tissue and cell types (Wiley et al., 1995b), suggesting that the restricted expression of the different TRAIL receptors regulates the induction of TRAIL-mediated apoptosis. However, messenger RNA for the four membrane-bound TRAIL receptors is also present in a wide range of normal cells and tissues, and each of the four TRAIL receptors bind TRAIL with comparable affinity (Degli-Esposti et al., 1997a; Degli-Esposti et al., 1997b). Therefore, the decoy receptors TRAIL receptor-3 and TRAIL receptor-4 were initially proposed to negatively regulate TRAIL signaling by competing with TRAIL receptor-1 and TRAIL receptor-2 for TRAIL binding (Marsters et al., 1997; Pan et al., 1997a; Pan et al., 1998; Sheridan et al., 1997). This hypothesis seemed plausible based on data from experiments utilizing TRAIL-sensitive cells overexpressing either TRAIL receptor-3 or TRAIL receptor-4, resulting in an inhibition of TRAIL-induced apoptotic cell death. Support for the protective role of TRAIL receptor-3 and/or TRAIL receptor-4 against TRAIL-induced tumor cell death was recently provided by results showing that TRAIL receptor-3 seems to be overexpressed in many primary tumors of the gastrointestinal tract (Sheikh et al., 1999). In addition, Sanlioglu et al. reported

that TRAIL receptor-4 expression correlated with TRAIL resistance in human breast tumor cell lines (Sanlioglu et al., 2005). Further studies from this group demonstrated that modulating TRAIL receptor expression, especially TRAIL receptor-4, on human lung and prostate tumor cells using small interfering RNA improved the tumoricidal activity of TRAIL (Aydin et al., 2007; Sanlioglu et al., 2007). Most recently, it was proposed that the ratio of TRAIL receptor-1 to TRAIL receptor-3 and TRAIL receptor-4 predicted the sensitivity of tumor cells to TRAIL-mediated apoptosis (Buneker et al., 2009). However, immunohistochemical analysis of several human tissues (brain, colon, heart, liver, lung, kidney, and testis) found TRAIL receptor-3 expression only within the brain, heart, liver, and testis (Spierings et al., 2004). Another study found no correlation between decoy receptor expression and TRAIL resistance in a panel of human melanoma cell lines, but a correlation between surface TRAIL receptor-1 and TRAIL receptor-2 expression and apoptosis was observed (Zhang et al., 1999). The authors suggested distribution of TRAIL receptors between the cytoplasm and the cell surface as a potential posttranslational regulatory mechanism. Thus, the premise of tumor cell sensitivity/resistance being controlled solely by TRAIL receptor-3/TRAIL receptor-4 expression is unlikely. Recently, it was found that expression of UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 14 (GALNT14) correlated with TRAIL sensitivity in a number of human tumor cell lines (Wagner et al., 2007). O-glycosylation of TRAIL receptor-1 and TRAIL receptor-2 promoted ligand-stimulated clustering of the receptor, which mediated the recruitment and activation of caspase-8. Further evidence demonstrating the ability of GALNT14 to sensitize tumor cells to TRAIL was obtained by RNA interference-mediated knock-down, which reduced cellular sensitivity to TRAIL-mediated killing.

Tumor cells contain multiple genetic mutations that account for the production of aberrant proteins, making it is likely that some tumors possess TRAIL receptor mutations that abrogate TRAIL receptor-mediated signaling. The *TRAIL receptor-2* gene has been mapped to human chromosome 8p21 (MacFarlane et al., 1997), a region that is frequently the site of losses of heterozygosity in many types of cancer (Pineau et al., 1999). Mutations of the *TRAIL receptor-2* gene have been identified in head and neck cancer, non-small cell lung cancer, breast cancer, non-Hodgkin's lymphoma, colorectal cancer, gastric cancer, and hepatocellular carcinoma (Arai et al., 1998; Jeng and Hsu, 2002; Lee et al., 1999; Lee et al., 2001; Pai et al., 1998; Park et al., 2001; Shin et al., 2001), and in each case the mutations were located within the death domain. The genes for the four TRAIL receptors are tightly clustered on human chromosome 8p21-22 (Degli-Esposti et al., 1997a; Degli-Esposti et al., 1997b; Walczak et al., 1997), suggesting they evolved relatively recently via gene duplication. It remains to be determined if the other TRAIL receptor genes are also mutated in some cancers that alter tumor cell sensitivity to TRAIL-induced apoptosis.

Another option for explaining the differences in tumor cell sensitivity to TRAIL-induced apoptosis relates to cell cycle progression. Apoptosis of dividing cells occurs at various stages of the cell cycle depending on the cell type and/or death-inducing stimulus. A study by Jin *et al.* determined, by using SW480 colon cancer and H460 lung cancer cell lines, that arresting these cells at the G₀/G₁ phase resulted in increased sensitivity to TRAIL-induced apoptosis compared to the same cells arrested at other cell cycle phases (Jin et al., 2002). The mechanism by which G₁-arrested cells display increased sensitivity to TRAIL remains to be determined. One possible explanation may lie in the levels of anti-apoptotic proteins during the different cell cycle phases. Though using a different system, Algeciras-Schimmich *et al.* (Algeciras-Schimmich et al., 1999) found that activated T cells arrested in G₁ phase contained high levels of cFLIP protein, which correlated with an increase in T cell receptor-induced apoptosis observed in other cell cycle phases. It is possible that cFLIP and/or other pro- or anti-apoptotic proteins fluctuate in a similar fashion in tumor cells.

The most attractive theory involves the differential expression of any of a number of pro- and anti-apoptotic proteins within the tumor cell that collectively help to regulate the signals generated from trimerized TRAIL receptor-1 and/or TRAIL receptor-2. Molecules such as cFLIP, Bcl-2 family members, inhibitor of apoptosis proteins, the extracellular signal-regulated kinases survival pathway, Akt, and Toso, have all been implicated in regulating the TRAIL receptor signal transduction pathway (Fulda et al., 2002a; Griffith et al., 1998; Griffith et al., 2002; Kim et al., 2008; Lee et al., 2006; Nesterov et al., 2001; Ng and Bonavida, 2002; Shiiki et al., 2000; Vaculova et al., 2006). At the level of the DISC, cFLIP inhibits caspase activation by competing for binding to FADD, thus acting as an important antiapoptotic factor (Irmeler et al., 1997). Suppression of cFLIP can be sufficient to sensitize some cancer cells to TRAIL-induced apoptosis (Geserick et al., 2008; Siegmund et al., 2002). High levels of XIAP expression have been observed in many tumor cell lines and may lead to TRAIL resistance by directly inhibiting caspases 3, 7 and 9, even in the presence of Smac/Diablo (Deveraux et al., 1997; Schimmer et al., 2004). Over-expression of anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-X_L, has also been shown to cause TRAIL resistance in type II cells (Hinz et al., 2000; Sinicrope et al., 2004). In addition, the anti-apoptotic Bcl-2 member, myeloid cell leukemia-1 protein (Mcl-1), can inhibit pro-apoptotic Bcl-2 family proteins such as Bid and its over-expression also confers resistance to TRAIL-mediated apoptosis *in vitro* (Clohessy et al., 2006). Interestingly, a recent report has suggested that some cancer cells may simultaneously exhibit more than one mechanism of resistance (Ndozangue-Touriguine et al., 2008).

5. Preclinical Studies

The majority of the studies published since the initial report describing TRAIL (Wiley et al., 1995b) have focused on the *in vitro* and *in vivo* tumoricidal activity of TRAIL. In these preclinical trials, recombinant human TRAIL (rhTRAIL) has induced apoptosis in multiple malignant cell lines, derived from both solid and hematologic malignancies, either alone or in combination with various chemotherapy agents or radiation (Ashkenazi et al., 1999; Gazitt, 1999; Kelley et al., 2001; Marini et al., 2005; Pollack et al., 2001). TRAIL receptors are widely expressed on the surface of somatic cells, which combined with the short distribution and elimination half-life of TRAIL, may significantly reduce the efficacy of TRAIL. One way to direct TRAIL to specific tumor cells involved the creation of a fusion protein of rhTRAIL and a CD19 single-chain Fv antibody fragment (Stieglmaier et al., 2008). This protein induced apoptosis in CD19 positive tumors, but normal blood cells were not affected. Cytotoxic agents and radiotherapy appear to sensitize cells to the effects of TRAIL (Chinnaiyan et al., 2000; Gliniak and Le, 1999). One possible mechanism for this phenomenon is the ability of p53 to induce the expression of both TRAIL receptor-1 and TRAIL receptor-2 (Liu et al., 2004; Takimoto and El-Deiry, 2000). Exposure to ionizing radiation has also been shown to upregulate TRAIL receptor-2 (Shankar et al., 2004). Thus, many tumors resistant to TRAIL-mediated apoptosis when TRAIL is used as a single agent will respond to a combination of conventional chemotherapy or radiotherapy plus TRAIL (Keane et al., 1999; Mizutani et al., 1999; Nimmanapalli et al., 2001).

A variety of anticancer agents have shown promise in preclinical trials when used in combination with TRAIL. The proteasome inhibitor bortezomib, approved for the treatment of multiple myeloma, appears to have a pro-apoptotic affect by inhibiting ubiquitin-mediated degradation of pro-apoptotic proteins, increasing p53 and TRAIL receptor-2 expression and decreasing cFLIP expression (Bonvini et al., 2007; Johnson et al., 2003; Sayers et al., 2003; Williams and McConkey, 2003). Bortezomib sensitizes some cell lines derived from breast, colon and kidney tumors to TRAIL-mediated apoptosis (Brooks et al., 2005). More recently, bortezomib has been shown to sensitize resistant non-small cell lung cancer cell lines to apoptosis induced by agonist monoclonal antibodies to TRAIL receptor-1 and TRAIL

receptor-2 (Luster et al., 2009). The kinase inhibitor sorafenib has been shown to down-regulate Bcl-xL, Mcl-1 and cFLIP and to potentiate TRAIL-induced cell death in several human leukemia cell lines but not in normal CD34⁺ bone marrow cells (Rosato et al., 2007). Histone deacetylase inhibitors are a new class of antitumor drugs that influence gene expression by promoting acetylation of histones (Johnstone, 2002), and have been found to upregulate TRAIL receptor-2 and sensitize various tumor cells to TRAIL-induced apoptosis (Fulda and Debatin, 2005b; Nakata et al., 2004; VanOosten et al., 2005a; VanOosten et al., 2005b). Moreover, HDAC inhibition can modulate TRAIL-induced apoptosis in tumor cells by other molecular mechanisms, including alteration of intracellular inhibitors or pro-apoptotic proteins (Fulda, 2008; Fulda and Debatin, 2005a).

Other reports have described reagents that target intracellular inhibitors of the apoptosis pathway. For example, the Bcl-2 inhibitor, BH3I-2', synergistically induces apoptosis in the presence of TRAIL in human prostate cancer cells (Ray et al., 2005). In TRAIL-resistant human colon cancer cells over-expressing Bcl-2, the Bcl-2 inhibitor, HA 14-1, similarly restores TRAIL sensitivity (Schimmer et al., 2004). Small interfering RNA-mediated inhibition of Bcl-XL enhances TRAIL-induced apoptosis (Zhu et al., 2005). Overexpression of Smac/DIABLO sensitizes neuroblastoma, melanoma, and glioma tumor cells to TRAIL-induced apoptosis, presumably by blocking the high levels of XIAP expressed in such tumors (Fulda et al., 2002b). Small molecule XIAP inhibitors, which were designed as capped tripeptides with unnatural amino acids based on the nuclear magnetic structure of a Smac peptide bound to XIAP, have also been shown to enhance TRAIL-induced apoptosis in mouse models by stimulating caspase activity (Deveraux et al., 1997; Li et al., 2004; Petrucci et al., 2007; Vogler et al., 2009).

Yet another method by which TRAIL can be used as an anti-tumor therapeutic lies in the delivery of the TRAIL coding sequence into the tumor cell. Our laboratory was the first to report the development of a nonreplicative recombinant adenoviral vector encoding the human TRAIL cDNA (Ad5-TRAIL) (Griffith et al., 2000). Infection of a tumor cell with Ad5-TRAIL leads to the rapid transcription and translation of the TRAIL cDNA into full-length membrane-bound protein that can trigger apoptosis. *In vivo*, TRAIL expression was observed up to seven days after an intratumoral injection of Ad5-TRAIL (Griffith and Broghammer, 2001), which is much longer than the 23-31 minute half-life of systemically administered rhTRAIL (Ashkenazi et al., 2008). Since our initial report, there have been a number of recombinant viral vectors containing the TRAIL cDNA described in the literature (Armeanu et al., 2003; Carlo-Stella et al., 2006; Dong et al., 2006; Griffith and Broghammer, 2001; Kagawa et al., 2001; Kock et al., 2007; Lee et al., 2002; Lillehammer et al., 2007; Seol et al., 2003; Voelkel-Johnson et al., 2002; Wenger et al., 2007; Yang et al., 2006; Zhang et al., 2008). Most recently, a recombinant fusion protein consisting of an endothelial growth factor receptor (EGFR)-directed antibody fragment fused to soluble TRAIL has been delivered via a replication-deficient adenovirus (Bremer et al., 2008). Mice with established intraperitoneal renal cell carcinoma xenografts that received a single intraocular injection of the virus experienced significant tumor load reduction and increased survival with no signs of hepatic toxicity despite predominant infection of the liver.

One intended use of Ad5-TRAIL is to directly kill tumor cells present at the injection site, but the intratumoral Ad5-TRAIL injection should theoretically generate sufficient apoptotic tumor cell debris to stimulate a systemic, tumor-specific immune response. Recent work from our laboratory investigated the ability of localized, intratumoral delivery of Ad5-TRAIL to induce systemic antitumor immunity using an experimental mouse model of transplantable renal cell carcinoma (VanOosten and Griffith, 2007). When Ad5-TRAIL was given intratumorally to mice bearing experimental renal cell carcinoma (Renca) tumors, only a minimal increase in survival and a low level of cytotoxic T lymphocyte activity was observed. An

immunostimulatory CpG oligodeoxynucleotide (CpG ODN) was then co-administered with the Ad5-TRAIL to enhance dendritic cell efficiency, which significantly augmented *in vivo* antigen-specific T cell proliferation and cytotoxic T lymphocyte activity, and prolonged animal survival. Interestingly, depletion of CD4⁺ or CD25⁺ cells prior to therapy further enhanced survival and *in vivo* cytotoxic T lymphocyte activity, suggesting a role for CD4⁺CD25⁺ regulatory T cells in preventing maximal immune stimulation. Tumor-free mice depleted of CD4⁺ cells were also able to reject a subsequent challenge of Renca cells, demonstrating the existence of immunologic memory. The pivotal role that CD4⁺ T cells play in the induction of CD8⁺ T cell responses has been highlighted in recent years (Albert et al., 2001; Bennett et al., 1997; Janssen et al., 2005; Schoenberger et al., 1998), where most CD8⁺ T cell-mediated responses depend on concomitant CD4⁺ T cell priming to be effective. Thus, we were surprised by the observation that CD4⁺ cell depletion prior to Ad5-mTRAIL/CpG ODN treatment augmented the CD8⁺ T cell response and significantly enhanced animal survival. Because of its profound immunostimulatory nature, we hypothesize that the CpG ODN used in our system substituted for the required CD4⁺ T cell help. The report by Cho et al. (Cho et al., 2000) in which CD8⁺ cytotoxic T lymphocyte activity was induced in CD4 or MHC class II-deficient animals supports this concept. These results demonstrated that local treatment with Ad5-TRAIL and CpG ODN (in our experimental setting) can augment tumor antigen cross presentation resulting in T cell proliferation, enhanced cytotoxic T lymphocyte activity, and increased animal survival. Current studies are assessing the Ad5-TRAIL/CpG ODN-induced immune response in an experimental model of metastatic cancer.

6. Clinical Trials

Translation of the preclinical observations of rhTRAIL or agonistic anti-TRAIL receptor monoclonal antibodies into the clinical realm has begun in recent years. A phase Ia trial of rhTRAIL consisting of a dose escalation to 15 mg/kg for patients with advanced solid tumors or non-Hodgkin's lymphoma has yielded encouraging results with no attributable toxicities (Herbst et al., 2006). Of the 32 patients evaluated, 17 (53%) had stable disease, 13 (41%) had progression and one patient with chondrosarcoma had a confirmed partial response. Preliminary results of a phase Ib trial of rhTRAIL in combination with rituximab in patients who have progressed after treatment for low-grade non-Hodgkin lymphoma have recently been reported (Yee et al., 2007). There were no dose-limiting toxicities or serious adverse events at doses of 4 and 8 mg/kg, and of the 5 patients assessed, 2 had stable disease, 1 had a partial response, and 2 had a complete response.

Clinical trials of agonistic anti-TRAIL receptor-1 and TRAIL receptor-2 monoclonal antibodies are also underway. In a phase I study of the TRAIL-R1 agonist mapatumumab, 49 patients with advanced solid malignancies safely received doses of up to 10 mg/kg every 14 days (Tolcher et al., 2007). The mean half-life of the antibody was 18 days. Nineteen patients had stable disease, with 2 instances lasting 9 months, but 2 patients did have elevated liver function tests probably related to mapatumumab administration. A phase Ib study of mapatumumab given to patients with advanced malignancies in combination with paclitaxel and carboplatin achieved a partial response in 4/28 patients (Chow et al., 2006). In another phase Ib study of mapatumumab with gemcitabine and cisplatin given to patients with advanced solid malignancies, doses as high as 30 mg/kg every 3 weeks were safe with 9/45 patients achieving a partial response and 13/45 patients experiencing stable disease after 6 cycles (Oldenhuis et al., 2008). A phase II trial of mapatumumab as a single agent was carried out in 32 previously treated patients with advanced non-small cell lung cancer. At a dose of 10 mg/kg given every 21 days, the antibody was well-tolerated and 9/32 patients had stable disease; however, none of the patients showed a response based on the Response Evaluation Criteria in Solid Tumors (RECIST) criteria (Greco et al., 2008).

A phase I trial of lexatumumab, a TRAIL receptor-2 agonistic antibody, in patients with advanced solid malignancies has been carried out with a dose of 10 mg/kg given every 21 days identified at the maximum tolerated dose (Plummer et al., 2007). The half-life of this antibody was 16 days, and 12 out of 37 patients had stable disease lasting a median of 4.5 months. Lexatumumab has also been evaluated in combination with gemcitabine, premetrexed, doxorubicin and FOLFIRI (leucovorin, fluorouracil and irinotecan) in a phase 1b study (Sikic et al., 2007). In this combination study, lexatumumab was well tolerated at 10 mg/kg with 3/41 patients experiencing a confirmed partial response.

Apomab is another agonistic antibody that interacts with an epitope on TRAIL receptor-2 that partially overlaps with both regions of the TRAIL binding site, and is capable of inducing TRAIL receptor-2 clustering and apoptosis (Adams et al., 2008). Apomab has undergone a phase I trial in patients with advanced, treatment-refractory solid tumors (Camidge et al., 2007). Doses up to 20 mg/kg given every 14 days were well tolerated. This antibody's half-life was 15-20 days, and no human anti-human antibodies were detected. There were no objective responses observed, but 1 patient with appendiceal cancer had stable disease and 1 patient with colorectal cancer had symptomatic improvement and shrinkage of target lesions.

We recently completed a Phase I clinical trial with Ad5-TRAIL in men with locally-confined prostate cancer, with the primary objectives of determining the toxicity profile and maximal tolerated dose. The ability of the vector to induce tumor cell death was also evaluated. Patients with histologically confirmed adenocarcinoma of the prostate [clinical stage T1c, T2a, T2b (as per American Joint Committee on Cancer 2002, 6th edition) all of whom were clinically N0, M0] and scheduled to undergo radical prostatectomy within 10 days following study entry were eligible for the study. As part of their staging evaluation, patients with a serum PSA >10ng/ml or tumors of Gleason grade >7 on biopsy also underwent a bone scan to rule out metastatic disease. All patients also underwent a staging endorectal MRI of the prostate prior to enrollment. Additional study inclusion criteria included the patients having no prior malignancies within the past 5 years with the exception of curatively treated basal cell or squamous cell carcinoma of the skin, an Eastern Cooperative Oncology Group performance status of 0 or 1, and no parenteral antibiotics < 7 days prior to study entry. Radiologic studies to document measurable or evaluable disease were done within 4 weeks prior to study entry. Each patient provided a signed informed consent form. Exclusion from the study occurred if there was a prior history of transurethral resection of the prostate, transurethral needle ablation of the prostate or microwave therapy to the prostate; a prior history of external beam radiation to the prostate or interstitial seed implantation (brachytherapy) to the prostate; a prior history of androgen ablation therapy for prostate cancer; a prior history of abdominoperineal resection or other procedure precluding patient from undergoing a transrectal ultrasound; a documented history of allergy to Gelfoam; or the presence of active infection.

We hypothesized that the intraprostatic delivery of Ad5-TRAIL could have two modes of action: direct infection of tumor cells resulting in tumor cell death and infection of normal prostate cells that are turned into "by-stander tumor killers." Still, adequate distribution of the vector throughout the prostate needed to be addressed to increase the efficacy of the Ad5-TRAIL therapy. Numerous substances have been used as carriers to enhance and sustain the delivery of soluble products to cancerous and normal tissues (Lee et al., 1997; Machan et al., 1997). One such agent is Gelfoam (Pfizer, Kalamazoo, MI), a collagen-based matrix that enhances the distribution and expression of virally-delivered genes when injected intratumorally (Siemens et al., 2000a; Siemens et al., 2000b). Gelfoam is able to absorb and hold within its interstices ~45 times its weight of blood and other fluids (Chemistry, 1947), and the absorptive capacity of Gelfoam is a function of its physical size, increasing with increasing gelatin volume (Goodman and Gliman, 1980). Gelfoam has been used for the delivery of soluble proteins and drugs, including antibiotics, and growth factors (Lee et al.,

1997; Park and Kim, 1997; Stepnick et al., 1995). Studies have evaluated the potential of Gelfoam to enhance intratumoral gene transfer and antitumor activity. Initial investigation showed Gelfoam enhanced canarypox (ALVAC) virus-mediated gene transfer (Fig. 3A) when compared with ALVAC injection in buffered saline (fluid phase). Subsequent studies were performed to compare adenoviral-mediated gene transfer to ALVAC with and without Gelfoam. As observed for ALVAC, Gelfoam also enhanced gene transfer with adenovirus suggesting the general applicability of the enhancement mechanisms (Fig. 3B). In addition, adenoviral transgene expression was significantly higher than ALVAC transgene expression. To further examine the Gelfoam-mediated enhancement of gene delivery, adenoviral transgene distribution in benign dog prostate was evaluated. Injection of Ad5- β -gal in phosphate buffered saline produced minimal β -gal expression, but Ad5- β -gal co-injected with Gelfoam resulted in an extensive expansion of β -gal expression (Fig. 3C). These data show that Gelfoam effectively enhances the distribution of virally transferred gene expression among prostate cells near the injection site, and suggest utility in gene therapy studies involving intraprostatic injections.

The vector was administered intraprostatically in a collagen (Gelfoam, 30 mg/ml) matrix to 12 patients using the following dose escalation: 1.3×10^8 pfu (4.2×10^9 particles); 4.2×10^8 pfu (1.3×10^{10} particles); 1.3×10^9 pfu (4.2×10^{10} particles); 4.2×10^9 pfu (1.3×10^{11} particles). For use in the delivery of Ad5-TRAIL, Gelfoam powder was mixed with sterile saline containing the appropriate dose of Ad5-TRAIL. The Gelfoam concentration was based on the fluid retention capacity, and not viral particle counts. Each group received 4 injections of Ad5-TRAIL in Gelfoam divided equally into both lobes of the prostate, further divided into 2 injection points in each lobe. The total volume injected was 2.5 ml. No adverse reactions were observed in any of the patients treated, and all patients tolerated the injection. Further, there were no complications with the surgical removal of the prostate after Ad5-TRAIL injection. A comparison of several surgical parameters compared to non-injected patients undergoing radical prostatectomy is shown in Table I.

Intraprostatic injection performed on the first 3 subjects also revealed the Ad5-TRAIL/Gelfoam mixture to be echogenic, allowing us to confirm its presence in the prostatic parenchyma (Fig. 3D). The clinical trial protocol specified that 3 subjects be enrolled at each of 4 dose levels with a 4 week break between dose levels to allow observation for side effects at each level. The Gelfoam was also detected by histological assessment in the injected prostate after prostatectomy (Fig. 4A). Inflammatory cells were evident in the areas where Gelfoam was present, as well as TUNEL-positive staining (Fig. 4B) – indicating DNA fragmentation indicative of apoptotic death.

7. Conclusions

TRAIL remains a promising biologically-targeted anti-cancer therapy that is currently in phase II clinical trials. TRAIL monotherapy may prove useful for those tumors that exhibit TRAIL sensitivity. However, research over the last decade has exposed the complexity of TRAIL signaling and a myriad of resistance mechanisms to TRAIL-induced killing that are present in many human tumor cells. Combination treatments to sensitize these cells to TRAIL are now the subject of new clinical trials.

Questions remain about the mechanisms that underlie TRAIL resistance in normal cells, and particular attention should be paid to hepatocytes given the known potential for TRAIL-induced toxicity in the liver. Understanding these mechanisms will be valuable in future developments in therapeutic applications of TRAIL for cancer treatment that will likely focus on overcoming TRAIL resistance.

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Figure 1.
TRAIL-TRAIL receptor system.

Figure 2.
Schematic representation of TRAIL-R1/-R2 apoptotic signaling pathway.

Figure 3. Effect of Gelfoam on viral distribution and transgene expression. **(A)** ALVAC- β -gal was injected into RM-1 tumors (400-600 mm³), which were removed 4 h later, fixed and stained for β -gal activity. **(B)** ALVAC-luc and Ad5-luc (10^7 pfu) were injected into RM-1 tumors (400-600 mm³), which were removed 24 h later, homogenized, and luciferase activity quantitated. **(C)** Effect of Gelfoam on adenovirus distribution in benign dog prostate. Ad5- β gal (10^7 pfu) was injected transperineally with ultrasound guidance. Prostates were removed 24 hr later, fixed and stained for β -gal. **(D)** Gelfoam is echogenic. TRUS images were taken of patients before and after receiving Ad5-TRAIL/Gelfoam.

Figure 4. Histological assessment of human prostate after Ad5-TRAIL/Gelfoam injection. **(A)** H&E staining of area where Gelfoam (amorphous purple component) was evident. **(B)** TUNEL staining of serial section.

Table I

Surgical comparison between prostate cancer patients receiving intraprostatic injections of Ad5-TRAIL and uninjected prostate cancer patients with similar disease stage.

	Ad5-TRAIL	Control	p value
LN dissection	2.33 (0.4)	2.0 (0-4)	
Peri-op bleeding	2.0 (1-4)	2.14 (1-4)	0.529
Inflam of anterior urethral stump	2.17 (1-4)	2.05 (104)	0.804
Gross local inflammation	2.0 (1-3)	2.05 (1-4)	0.804
Diff of NS	1.75 (0-3)	2.33 (1-3)	0.382
Ability to dissect prostate	2.17 (1-4)	2.24 (1-4)	0.805
Total est. blood loss (ml)	720.83 (400-1500)	896.05 (450-2000)	0.115
Diff of anastomosis	2.0 (1-3)	2.24 (1-4)	0.621