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The TRAIL to targeted therapy of breast cancer

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Abstract

Breast cancers can be classified into those which express the estrogen (ER) and progesterone (PR) receptors, those with HER-2 amplification, and those without expression of ER, PR, or amplified HER-2 (referred to as triple-negative or basal-like breast cancer). Tumor Necrosis Factor Apoptosis Inducing Ligand (TRAIL) activates apoptosis upon binding to its receptors in many tumor types and the ligand and agonist antibodies are currently being studied in patients in clinical phase I and phase II trials. Cell line studies suggest that many breast cancer cell lines are very resistant to TRAIL-induced apoptosis. However, recent data suggest that a subset of triple-negative/basal-like breast cancer cells is sensitive to TRAIL as a single agent. In addition many studies have demonstrated that resistance to TRAIL-mediated apoptosis in breast cancer cells can be overcome by combinations of TRAIL with chemotherapy, radiation, and various targeted agents. This review will discuss the current understanding of the mechanisms which control TRAIL-mediated apoptosis in breast cancer cells. The preclinical data supporting the use of TRAIL ligands and agonistic antibodies alone and in combination in breast cancer will also be discussed.

I. INTRODUCTION

Breast cancer is the most frequently diagnosed cancer in women and one of the leading causes of cancer death for women. Worldwide, over 1.3 million cases of invasive breast cancer are diagnosed, and more than 450,000 women die from breast cancer annually (Garcia *et al.*, 2007). In the US, approximately 180,000 cases of invasive breast cancer and 60,000 cases of *in situ* breast cancer are diagnosed annually, and more than 40,000 women die from breast cancer each year – second only to lung cancer (Jemal *et al.*, 2008). The mortality due to breast cancer has been declining in the US since 1990. The death rate was 32.69 per 100,000 women in 1991 but fell to 25.19 per 100,000 women in 2003 (Jemal *et al.*, 2007). The continuing decrease in mortality from breast cancer has been attributed to early detection due to screening, improved adjuvant therapy, and more recently to decreases in the incidence due to lowered rates of usage of hormone replacement therapy (Berry *et al.*, 2005; Ravdin *et al.*, 2007).

Breast cancer can be divided into several distinct subtypes that have prognostic and therapeutic implications. Clinically, breast cancer patients routinely have the expression of estrogen receptor (ER), progesterone receptor (PR), and amplification of HER-2 evaluated (Brenton *et al.*, 2005). This allows the classification of breast cancer as hormone receptor positive tumors, HER-2 amplified tumors (which may or may not express hormone receptors), and those tumors which do not express ER, PR, and do not have HER-2 amplification. The latter group is referred to as triple-negative breast cancer based on the lack of these three molecular markers. Generally, hormone receptor expressing breast cancers have a more favorable prognosis than either those with HER-2 amplification or those that are triple-negative (Brenton *et al.*, 2005). While all breast tumor types may be

treated with chemotherapy, therapeutic options in both early and late stage breast cancer are affected significantly by the expression of these three markers. Tumors that express ER and PR are treated with agents that interfere with hormone production or action. Tumors that have amplified HER2 are treated with agents that inhibit HER-2. Triple-negative tumors are treated with predominantly chemotherapy (Brenton *et al.*, 2005).

Recent expression profiling of human breast cancers has allowed classification of the tumors based on clustering and the similarity of expression patterns between normal breast cells and tumors (Perou *et al.*, 2000; Sorlie *et al.*, 2001). The hormone receptor expressing breast cancers resembled most closely the luminal cells of the breast ducts but could be further subdivided into several subgroups that have different prognoses and responses to hormonal therapy. The tumors with HER-2 amplification clustered together and were found to have a poorer prognosis than the luminal subtype. These data were compiled prior to the introduction of trastuzumab. The triple-negative tumors resembled most closely basal cells, cells found on the outside of the breast ducts, and had the worst prognosis (Perou *et al.*, 2000; Sorlie *et al.*, 2001). Subsequent analyses have suggested that the clinical triple-negative classification and the array based basal classification significantly overlap but are not identical (Rakha *et al.*, 2009). Ongoing clinical trials are beginning to evaluate the use of these and other molecular classifications of breast cancer for making treatment decisions (Sotiriou and Pusztai, 2009). While yet to be applied to the routine care of breast cancer patients, array based molecular classification is likely to allow more individualized treatment in the future.

Despite the advances made in the detection and treatment of early breast cancer that have contributed to the declining mortality in the US, metastatic breast cancer remains an incurable disease. More efficacious treatments to prevent relapse in early stage patients and to treat metastatic disease are needed if a major impact is to be realized in the mortality of breast cancer. This review will focus on the potential use of Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL) receptor agonists for the treatment of breast cancer.

II. TRAIL AND ITS RECEPTORS

There are several fundamental apoptosis pathways in cells which are defined by where the initial caspase activation occurs (Fig. 1). One pathway, referred to as the extrinsic pathway, mediates activation of caspase 8 or caspase 10 by ligand binding to cell surface receptors (Ashkenazi, 2002; Danial and Korsmeyer, 2004). A second pathway, referred to as the intrinsic pathway, mediates caspase 9 activation by the mitochondrial release of proapoptotic proteins such as cytochrome c in response to a variety of stimuli such as the absence of growth factors, DNA damage, and viral infection (Danial and Korsmeyer, 2004). Cytosolic cytochrome c binds to apoptotic peptidase activating factor 1 (APAF-1) and activates caspase 9 in an ATP dependent reaction (Danial and Korsmeyer, 2004). Other pathways trigger the activation of caspase 2 in response to heat shock or DNA damage (Sidi *et al.*, 2008; Tu *et al.*, 2006) or caspase 12 in response to ER stress (Nakagawa *et al.*, 2000). After the activation of the initiator caspases, the pathways converge on downstream caspases such as caspase 3, caspase 6 and caspase 7, so-called effector caspases (Danial and Korsmeyer, 2004). In addition, after the activation of the primary initiator caspases specific to each pathway, other initiator caspases can be activated downstream of these primary caspases (Fig. 1). For example, death receptor activated caspases 8 and 10 can cleave the BH3 only protein BID, leading to its translocation to the mitochondria where it activates the mitochondrial pathway leading to activation of caspase 9 (Ashkenazi, 2002; Suliman *et al.*, 2001). Similarly, activated caspase 3 can directly activate the death receptor initiator caspase 8 (Slee *et al.*, 1999; Sun *et al.*, 1999). Thus, while each pathway is defined by the initiating

stimuli and caspase that becomes activated, these pathways form an interconnected network within the cell.

The death receptors belong to the Tumor Necrosis Factor (TNF) family. The TNF family has more than 20 receptors of which six are death receptors (DR) and activate apoptosis in response to binding of their respective ligands (Ashkenazi, 2002). The six proteins are TNFR1 (a.k.a. Death Receptor 1 or DR1), FAS (a.k.a. CD95, DR2), DR3, TRAIL-R1, TRAIL-R2, and DR6. These receptors are activated by their respective ligand: TNF for TNFR1, CD95 Ligand for FAS (a.k.a FAS Ligand), TL1A for DR3, and TRAIL for TRAIL-R1 and TRAIL-R2 (Ashkenazi, 2002). A ligand for DR6 has not been identified. All of these receptors are homotrimeric proteins which activate apoptosis via a cytoplasmic domain known as the death domain (DD) (Fig. 2). These domains serve as protein dimerization motifs that, upon ligand binding, recruit the DD containing adaptor FAS-Associated via Death Domain (FADD) protein. FADD in turn recruits caspases 8 and/or 10 via a death effector domain (DED), a second protein dimerization motif. Recruitment of FADD and the caspases to the receptor forms the death inducing signaling complex (DISC) and results in activation of the initiator caspases (caspase 8 and caspase 10). The initiator caspases exist in the cell as inactive proenzymes which become activated upon dimerization at the DISC. They subsequently undergo auto-processing resulting in the release of a large subunit and a small subunit from the precursor. The processed caspases form a tetramer composed of two large subunits and two small subunits and have markedly increased activity compared to the unprocessed enzyme (Ashkenazi, 2002; Riedl and Shi, 2004). Once activated the initiator caspases can directly cleave and activate the downstream effector caspases. Also, activated caspase 8 and caspase 10 can cleave the BH3 only containing protein Bid, which then translocates to the mitochondrial membrane where it activates the intrinsic pathway (Fig. 2). Cellular FLICE inhibitory protein (cFLIP) is an important negative regulatory molecule in the death receptor pathway. cFLIP was identified by homology to viral FLIP proteins which inhibit apoptosis by binding to the DR/FADD complex via DED domains and preventing recruitment of caspases 8 or 10 (Irmeler *et al.*, 1997). cFLIP is similar in structure to the caspase 8 proenzyme, containing two N-terminal DED domains and caspase related domains in the C-terminal. However, the active site cysteine required for caspase activity is replaced by a tyrosine in cFLIP. Thus, cFLIP can be recruited to the DISC and prevent the initiator caspases from being recruited and activated (Fig. 2).

Also, activation of DRs can result in signaling that does not induce apoptosis. For example, TNFR1 can, via recruitment of TRADD, regulate gene expression by activation of Nuclear Factor-kappa B (NF- κ B) and AP1 transcription factors (Wilson *et al.*, 2009). Similarly, FAS can activate proinflammatory responses in addition to apoptotic signaling (Wilson *et al.*, 2009). Like TNFR1, TRAIL receptors can activate NF- κ B. This appears to be mediated by the recruitment of RIP which leads to activation of the inhibitor of κ B kinases, phosphorylation of the inhibitor of κ B, and activation of NF- κ B (Falschlehner *et al.*, 2007). Also, TRAIL can activate AKT and MAPK, but the links to these pathways are unclear (Falschlehner *et al.*, 2007). While the TRAIL receptors can signal to non-apoptotic pathways, this review will focus on the role of the TRAIL ligand and its DRs in inducing apoptosis in breast cancer cells.

TRAIL (a.k.a. Apo2L) was initially identified and cloned based on homology searches of EST databases for cDNAs related to TNF and Fas ligand (Pitti *et al.*, 1996; Wiley *et al.*, 1995). These studies identified a ligand that is highly homologous to FAS and TNF and that is able to induce apoptosis in a diverse range of tumor cell lines. Also, the receptors for TRAIL were identified based on homology searches for ESTs that were similar to TNFR1 (Pan *et al.*, 1997a; Pan *et al.*, 1997b). In humans, there are two receptors for TRAIL that can induce apoptosis upon ligand binding, TRAIL-R1 (a.k.a. DR4) and TRAIL-R2 (a.k.a. DR5,

TRICK2, and KILLER) (Fig. 3) (MacFarlane *et al.*, 1997; Pan *et al.*, 1997a; Pan *et al.*, 1997b; Screaton *et al.*, 1997; Sheridan *et al.*, 1997; Walczak *et al.*, 1997; Wu *et al.*, 1997). There are three receptors, TRAIL-R3 (a.k.a. Decoy Receptor 1, TRID, and LIT), TRAIL-R4 (a.k.a. Decoy Receptor 2 and TRUNND), and TRAIL-R5 (a.k.a. osteoprotegerin) which have incomplete death domains or lack death domains (Degli-Esposti *et al.*, 1997a; Degli-Esposti *et al.*, 1997b; Emery *et al.*, 1998; Marsters *et al.*, 1997; Pan *et al.*, 1997a; Pan *et al.*, 1998; Schneider *et al.*, 1997a; Sheridan *et al.*, 1997). These three receptors act as inhibitors of TRAIL-induced apoptosis by binding the ligand and sequestering it from the death inducing receptors (Fig. 3). Expression of TRAIL and TRAIL receptors is found widely distributed throughout the organism (Spierings *et al.*, 2004). Animal studies implicate TRAIL and its receptor as negative regulators of immune responses. TRAIL deficient mice have a defect in thymocyte apoptosis and a concomitant hypersensitivity to the development of autoimmune T-cell mediated responses in several experimental systems (Cretney *et al.*, 2005; Lamhamedi-Cherradi *et al.*, 2003). Also, there is evidence that TRAIL may enhance T-cell mediated neural cell death in an animal model of autoimmune encephalomyelitis (Aktas *et al.*, 2005). Thus, TRAIL may both inhibit and promote autoimmune disease. Mice have a homologue of TRAIL-R2 but do not have a TRAIL-R1 (Kelley and Ashkenazi, 2004). Loss of TRAIL-R2 results in enhanced immune responses to CMV infection, consistent with a role as a negative regulator of innate immune responses (Diehl *et al.*, 2004). Importantly, animal studies suggest that TRAIL plays a role in tumor surveillance. Neutralization or deletion of TRAIL in several animal models demonstrates that the loss of TRAIL activity promotes the growth and metastasis of tumors in both transplanted and spontaneous tumors (Cretney *et al.*, 2002; Sedger *et al.*, 2002; Takeda *et al.*, 2001; Zerafa *et al.*, 2005). The anti-tumor effects of TRAIL in these studies appear to be mediated by NK cells (Cretney *et al.*, 2002; Takeda *et al.*, 2001). In addition T-cell mediated graft versus tumor activity appears to be mediated at least in part by TRAIL as allogeneic hematopoietic-cell transplantation from TRAIL deficient animals resulted in less graft versus tumor activity (Schmaltz *et al.*, 2002).

The interest in the anti-tumor activity of TNF family ligands is based in part on work done by William Coley over 100 years ago which found that bacterial toxins could induce hemorrhagic necrosis of tumors and induce meaningful responses in patients with inoperable tumors such as sarcomas (Coley, 1893; Coley, 1906). The search for the biological mediators of these responses led to the identification of TNF and its receptor (Balkwill, 2009; Carswell *et al.*, 1975). Inspired by the results reported by Coley, TNF was tested in patients with cancer but TNF causes severe toxicity and has little efficacy as systemic therapy for cancer (Balkwill, 2009). The second TNF family DR ligand, FAS ligand, has not been tested in clinical trials due to lethal hepatic apoptosis in animal studies (Ogasawara *et al.*, 1993). TRAIL is currently in clinical trials and has generated much excitement as a potential systemic cancer therapy. Early *in vitro* experiments suggested that TRAIL could kill tumor cells in culture but was not toxic to nontransformed cells (Ashkenazi *et al.*, 1999; Keane *et al.*, 1999; Keane *et al.*, 2000). Subsequent experiments with TRAIL in mice, cynomolgus monkeys, and chimpanzees confirmed that TRAIL is well tolerated by animals (Ashkenazi *et al.*, 1999; Kelley *et al.*, 2001; Lawrence *et al.*, 2001). This has led to phase I clinical trials of both TRAIL and agonistic TRAIL receptor antibodies which have demonstrated that these agents are well tolerated at doses that result in serum levels that are above the therapeutic concentrations that have been used in preclinical studies (Fig. 4) (Camidge *et al.*, 2007; Hotte *et al.*, 2008; Ling *et al.*, 2006; Plummer *et al.*, 2007; Tolcher *et al.*, 2007). These agents are undergoing further testing in clinical trials as single agents and in combination with chemotherapy.

III. TRAIL-INDUCED APOPTOSIS IN BREAST CANCER CELLS

TNF and FAS agonists have been studied using *in vitro* models of breast cancer (*e.g.*, Jaattela *et al.*, 1995; Keane *et al.*, 1996). However, the toxicity and lack of efficacy of TNF in clinical trials and the toxicity of FAS ligands in preclinical studies has precluded further clinical development of these ligands (Balkwill, 2009; Ogasawara *et al.*, 1993). Initial studies of TRAIL-mediated apoptosis in breast cancer cell lines demonstrated that while TRAIL could induce apoptosis in the MDA-MB-231 breast cancer cell line, the majority of cell lines tested were very resistant to TRAIL-mediated apoptosis (Ashkenazi *et al.*, 1999; Buchsbaum *et al.*, 2003; Keane *et al.*, 1999; Keane *et al.*, 2000; Singh *et al.*, 2003). These studies were able to establish that TRAIL induced caspase mediated apoptosis in the sensitive cell line and that TRAIL activated caspases within minutes of addition to the cells (Keane *et al.*, 1999; Keane *et al.*, 2000). However, these studies did not systematically evaluate breast cancer cell lines with different phenotypes as defined above (*e.g.*, hormone receptor positive, HER-2 amplified, or triple-negative cell lines). Recently, our laboratory reexamined TRAIL-sensitivity in breast cancer cells using a panel of cell lines that included multiple cell lines of each phenotype (Neve *et al.*, 2006; Rahman *et al.*, 2009). This study found that TRAIL sensitivity varied with the phenotype of the breast cancer cell lines (Fig. 5) (Rahman *et al.*, 2009). Strikingly, eight of eleven triple-negative breast cancer cell lines were very sensitive to TRAIL-induced apoptosis with the IC₅₀ ranging from 10–250 ng/ml (~0.2–5.8 nM). By contrast all five of the ER positive cell lines tested were resistant to TRAIL-induced apoptosis across a wide range of doses. Two of five cell lines with HER-2 amplification showed a modest sensitivity to TRAIL, only reaching an IC₅₀ at approximately 1000 ng/ml (~20 nM). Other studies, although not designed to specifically look at TRAIL sensitivity based on the phenotype of the cell lines, found similar results. For example, Chinnaiyan *et al.* studied TRAIL sensitivity in 10 breast cancer cell lines (Chinnaiyan *et al.*, 2000). They found that three of five triple-negative breast cancer cell lines were TRAIL-sensitive. Two HER-2 amplified breast cancer cell lines and three ER positive cell lines were TRAIL-resistant (Chinnaiyan *et al.*, 2000). Similarly, Buchsbaum *et al.* found that an agonistic anti-TRAIL-R2 antibody induced apoptosis in one of two triple-negative breast cancer cell lines but not any of four HER-2 amplified breast cancer cell lines nor in an ER positive cell line (Buchsbaum *et al.*, 2003). Together the data from these three studies demonstrated that 10 of 14 triple-negative breast cancer cell lines were sensitive to TRAIL-induced apoptosis while only two of eight HER-2 amplified cell lines, and none of seven ER positive lines were sensitive to TRAIL-induced apoptosis.

In cancer cells, the mutation or absence of p53 renders cells resistant to chemotherapy or radiation therapy (Bunz *et al.*, 1999; Lee and Bernstein, 1993; Lowe *et al.*, 1994; Lowe *et al.*, 1993). In breast cancer specifically, primary resistance to doxorubicin has been associated with p53 mutations (Aas *et al.*, 1996). Interestingly, recent work has found that p53 absence or mutation is frequent in triple-negative/basal-like breast cancers (Brenton *et al.*, 2005). The TRAIL-sensitive triple-negative breast cancer cell lines described above frequently have lost or mutated p53 (Neve *et al.*, 2006; Rahman *et al.*, 2009). The ability of TRAIL to kill tumors that are p53 mutant or deleted has been observed in cell lines from a wide variety of tumor types (Ashkenazi *et al.*, 2008). This suggests that TRAIL ligands may be particularly useful as a therapeutic agent in tumors deficient in p53.

Recent work has classified a large number of breast cancer cell lines based on transcriptional profiling (Neve *et al.*, 2006). Like the array based profiling of primary tumors described above (Perou *et al.*, 2000; Sorlie *et al.*, 2001), breast cancer cell lines could be classified into two main groups, luminal and basal. The triple-negative breast cancer cell lines were classified as basal by this analysis (Neve *et al.*, 2006). The basal group was further subdivided into basal “A” and basal “B” groups. The Basal B cell lines were distinguished

based on expression of mesenchymal markers such as the cytoskeletal protein vimentin. An independent group classified breast cancer cell lines by transcriptional profiling and similarly found that a subset of the triple-negative cell lines had mesenchymal features (Charafe-Jauffret *et al.*, 2006). Interestingly, we found that all of the TRAIL-sensitive triple-negative cell lines we tested have mesenchymal features based on these analyses (Charafe-Jauffret *et al.*, 2006; Neve *et al.*, 2006; Rahman *et al.*, 2009). In contrast, the TRAIL-resistant triple-negative cell lines in our study were ones that are classified as epithelial by these analyses (Charafe-Jauffret *et al.*, 2006; Neve *et al.*, 2006; Rahman *et al.*, 2009). A number of studies using the mesenchymal triple-negative breast cancer cell line MDA-MB-231 have demonstrated the efficacy of TRAIL ligands or agonistic antibodies in xenograft studies, confirming the sensitivity of this cell line *in vivo* (Buchsbaum *et al.*, 2003; Shankar *et al.*, 2004; Singh *et al.*, 2003; Thai le *et al.*, 2006). Together these results suggest that triple-negative/basal-like breast cancers with mesenchymal features are more likely to be sensitive to TRAIL-induced apoptosis.

The mesenchymal characterization of the triple-negative breast cancer cell lines which are sensitive to TRAIL was initially identified by transcriptional profiling and confirmed by immunoblotting for vimentin, a mesenchymal marker protein (Charafe-Jauffret *et al.*, 2006; Neve *et al.*, 2006; Rahman *et al.*, 2009). However, the mesenchymal subset of tumors was not identified in the early transcriptional profiling of primary breast cancer samples that defined the luminal and basal subsets of breast cancer (Sorlie *et al.*, 2001). More recently, immunohistochemical studies of primary breast tumors have identified a subset of tumors in which the cancer cells express vimentin, consistent with the existence of mesenchymal tumors (Livasy *et al.*, 2006; Umemura *et al.*, 2005; Willipinski-Stapelfeldt *et al.*, 2005). The largest study of more than 2500 primary breast tumors found that approximately 14% of all of the tumors and 35% of the ER negative tumors expressed vimentin (Willipinski-Stapelfeldt *et al.*, 2005). In this study, approximately 7% of the ER positive tumors expressed vimentin. The enrichment of vimentin positive tumors within the ER negative samples is consistent with an enrichment within the triple-negative samples, but this study did not simultaneously evaluate HER-2 amplification so that vimentin positive tumors cannot be classified as triple-negative (Willipinski-Stapelfeldt *et al.*, 2005). The other two studies identified vimentin expression in 17 of 18 and 4 of 11 triple-negative breast cancer samples (Livasy *et al.*, 2006; Umemura *et al.*, 2005). In the study by Livasy *et al.*, the tumors were categorized as luminal, basal, or HER-2 amplified by cDNA microarray expression profiling (Livasy *et al.*, 2006). This study found 17 of 18 triple-negative/basal-like tumors had strong and diffuse vimentin staining in the tumor cells (Livasy *et al.*, 2006). Only 1 of 16 ER positive/luminal cancers and 1 of 12 HER-2 amplified tumors expressed vimentin in the tumor cells. These studies suggest that a subset of triple-negative breast cancers have mesenchymal features.

As described above, in humans there are two receptors for TRAIL that induce apoptosis, TRAIL-R1 and TRAIL-R2. Previous work using mutants of TRAIL that bind selectively to either TRAIL-R1 or TRAIL-R2 has demonstrated that TRAIL induces apoptosis predominantly via TRAIL-R1 in some tumor types and via TRAIL-R2 in others (Kelley *et al.*, 2005; MacFarlane *et al.*, 2005; van der Sloot *et al.*, 2006). Both receptors are expressed at the mRNA and protein levels in the TRAIL-sensitive breast cancer cells (Keane *et al.*, 1999; Rahman *et al.*, 2009). A study using agonist antibodies to either TRAIL-R1 or TRAIL-R2 has shown that both can induce apoptosis in the MDA-MB-231 cell line (Zhang and Zhang, 2008). Interestingly, despite expression of both receptors on the breast cancer cells and the ability of the agonist anti-TRAIL-R1 antibody to induce apoptosis, experiments using RNA interference or receptor selective mutants of TRAIL indicate that TRAIL-R2 is the predominant mediator of apoptosis in the breast cancer cells exposed to TRAIL (Kelley *et al.*, 2005; Rahman *et al.*, 2009). The basis for the selective activity of TRAIL-R2 in the

breast cancer cells is not clear. One possibility is that the absolute level of TRAIL-R2 at the cell surface is significantly greater than that of TRAIL-R1, but this has not been demonstrated. Alternatively, binding studies suggest that TRAIL-R2 has a significantly higher affinity ($K_d = 2$ nM) for TRAIL than TRAIL-R1 ($K_d = 70$ nM) when the binding studies are carried out at 37°C (Truneh *et al.*, 2000). This observation could explain the discordant results described above, where the agonist anti-TRAIL-R1 antibody can induce apoptosis in MDA-MB-231 cells but the ligand utilizes preferentially TRAIL-R2.

Overall, these observations about breast cancer subtype and receptor selectivity will be important in planning clinical trials of TRAIL ligands or agonistic antibodies in breast cancer patients.

IV. MECHANISMS DETERMINING TRAIL SENSITIVITY IN BREAST CANCER CELLS

The underlying determinants of TRAIL sensitivity in the breast cancer cell lines have not been clearly established. While the experiments described above suggest a subset of breast cancer cells are intrinsically more sensitive to TRAIL (*i.e.*, triple-negative breast cancer cells with mesenchymal features), no clear mechanistic basis for this was determined (Rahman *et al.*, 2009).

Of the five receptors for TRAIL two receptors, TRAIL-R1 and TRAIL-R2, induce caspase activation and apoptosis upon ligand binding (Fig. 3) (MacFarlane *et al.*, 1997; Pan *et al.*, 1997a; Pan *et al.*, 1997b; Screaton *et al.*, 1997; Sheridan *et al.*, 1997; Walczak *et al.*, 1997; Wu *et al.*, 1997). In some tumor types, such as neuroblastoma, lack of surface expression of TRAIL-R1 or TRAIL-R2 has been found to correlate with the lack of TRAIL sensitivity (Yang *et al.*, 2003). Expression of TRAIL receptors on breast cancer cells has been examined in a number of studies. The levels of receptor either by mRNA, total protein levels, or surface expression are not predictive of TRAIL-sensitivity (Buchsbbaum *et al.*, 2003; Keane *et al.*, 1999; Rahman *et al.*, 2009). For example, we determined the surface expression of both TRAIL-R1 and TRAIL-R2 in seven sensitive and seven resistant breast cancer cell lines (Fig. 6). The expression of TRAIL-R1 overlapped significantly between the sensitive and resistant cell lines and did not allow discrimination of the sensitive and resistance cells. However, as described above, studies suggest that TRAIL-R1 may not contribute significantly to the induction of apoptosis by TRAIL in breast cancer cells (Kelley *et al.*, 2005; Rahman *et al.*, 2009). While the expression of TRAIL-R2 is generally higher on TRAIL-sensitive cells than on TRAIL-resistant cells there was again significant overlap between the surface level on sensitive and resistant cells (Fig. 6). In addition, many of the TRAIL-resistant cells expressed only marginally less surface TRAIL-R2 than the sensitive cells (Fig. 6). Three receptors, TRAIL-R3, TRAIL-R4, and TRAIL-R5, can bind TRAIL but do not have a functional death domain (Fig. 3) (Degli-Esposti *et al.*, 1997a; Degli-Esposti *et al.*, 1997b; Emery *et al.*, 1998; Marsters *et al.*, 1997; Pan *et al.*, 1997a; Pan *et al.*, 1998; Schneider *et al.*, 1997a; Sheridan *et al.*, 1997). These receptors have been shown to inhibit TRAIL-induced apoptosis when overexpressed and have been called decoy receptors. However, the expression of these decoy receptors has not been found to correlate with resistance to TRAIL-induced apoptosis in breast cancer cell lines or in other tumors (Griffith *et al.*, 1998; Griffith *et al.*, 1999; Keane *et al.*, 1999; Rahman *et al.*, 2009). Thus expression levels of TRAIL receptors do not appear to be predictive of TRAIL sensitivity.

Examination for evidence of mutation in the death inducing TRAIL receptors (TRAIL-R1 and TRAIL-R2) in breast cancer cells has given contradictory results. One study found that 3 of 57 (5.3%) primary breast cancers had mutations in the death domain of TRAIL-R1 and 4 of 57 (7.0%) had mutations in the death domain of TRAIL-R2 (Shin *et al.*, 2001).

Interestingly, all of these mutations were found in the group of 34 breast cancers that were metastatic to the regional lymph nodes and none were found in the 23 samples from tumors that had not spread to regional nodes. In addition, these mutant receptors were impaired in their ability to induce apoptosis compared to wild type receptors (Shin *et al.*, 2001). A second study looked at a series of primary breast cancers and found that 2 out of 50 had sequence variants in TRAIL-R1 and 11 of 95 tumors had sequence variants in TRAIL-R2. However, all of the sequence variants were found in matched normal tissue leading to the conclusion that they represented polymorphisms and not cancer specific mutations. None of these sequence variants were in the death domain but no functional studies were undertaken for these sequence variants. Overall, the second study concluded that there was no relationship between these polymorphisms and breast cancer. While no systematic sequence data for the cell lines has been reported, one study has sequenced TRAIL-R1 and TRAIL-R2 from seven breast cancer cell lines but found no correlation of sequence variants with TRAIL sensitivity (Zhang and Zhang, 2008). Similarly, we have examined the mRNA sequence for TRAIL-R1 and TRAIL-R2 from a number of the TRAIL-sensitive and resistant cells and have not found evidence for mutation or sequence variation that correlates with TRAIL sensitivity or resistance (unpublished data).

In general, studies have not identified individual components of either the TRAIL pathway (*e.g.*, TRAIL receptors, FADD, caspase 8) or apoptosis modulators (*e.g.*, cFLIP, IAPs, or Bcl-2 family members) whose expression is predictive of TRAIL sensitivity or resistance (Keane *et al.*, 1999; Rahman *et al.*, 2009). A number of studies have found that altering the levels or activity of antiapoptotic proteins such as Bcl-2 or Bcl-XL, FLIP, NF κ B or Survivin can alter the sensitivity of cells to TRAIL (Fulda and Debatin, 2004; Fulda *et al.*, 2002; Guseva *et al.*, 2008; Keane *et al.*, 2000; Kim *et al.*, 2003; Palacios *et al.*, 2006). However, these studies do not demonstrate that the levels or activity of these proteins are the primary reason for TRAIL resistance in the breast cancer cells.

Recent analysis in pancreatic cancer, colorectal cancer, non-small-cell lung cancer, and melanoma cell lines has identified low expression of O-glycosylation genes as a potential mechanism of TRAIL-resistance (Wagner *et al.*, 2007). This study found that O-glycosylation of the TRAIL receptors promoted ligand induced clustering of the receptors and subsequent recruitment and activation of initiator caspase 8. However, gene expression analysis in the breast cancer cell lines did not find a correlation between the genes which regulate O-glycosylation and TRAIL sensitivity (Rahman *et al.*, 2009). Further experiments with inhibitors of O-glycosylation or overexpression of genes which mediate O-glycosylation did not affect TRAIL sensitivity in the breast cancer cell lines (unpublished observations). Thus, this mechanism does not appear to determine TRAIL sensitivity in breast cancer cells.

An intriguing observation is that the TRAIL-sensitive MDA-MB-231 breast cancer cell line (a triple-negative breast cancer cell line with basal and mesenchymal features) has low expression of the small heat shock protein, α B-crystallin, while several TRAIL-resistant cell lines (including a TRAIL-resistant triple-negative and an ER positive cell line) have high expression of α B-crystallin (Kamradt *et al.*, 2005). Overexpression of α B-crystallin in MDA-MB-231 decreases the sensitivity to TRAIL and RNAi mediated knockdown of α B-crystallin in one cell line increased sensitivity to TRAIL (Kamradt *et al.*, 2005). No systematic evaluation of α B-crystallin and TRAIL sensitivity in a more extensive panel of breast cancer cells representing the different subtypes has been undertaken. Paradoxically, studies of primary breast cancer samples have demonstrated that α B-crystallin is expressed predominantly in triple-negative/basal-like breast cancers and not in ER positive or HER-2 positive tumors (Moyano *et al.*, 2006; Sitterding *et al.*, 2008). These results are at odds with the cell line data described above which showed low expression in one triple-negative/basal-

like breast cancer cell line (MDA-MB-231) but high expression in one triple-negative/basal-like and one ER positive cell line (MDA-MB-468 and MCF 7 respectively) (Kamradt *et al.*, 2005). Thus, further work will be needed to determine if α B-crystallin is predictive of TRAIL sensitivity in a wider sample of breast cancer cells.

Like many cell surface receptors, death receptors undergo activation induced internalization via the endocytic pathway (Austin *et al.*, 2006; Higuchi and Aggarwal, 1994; Kohlhaas *et al.*, 2007; Lee *et al.*, 2006; Schneider-Brachert *et al.*, 2004; Siegel *et al.*, 2004). Internalization appears required for optimal induction of apoptosis by TNFR and FAS (Lee *et al.*, 2006; Schneider-Brachert *et al.*, 2004). Studies with TRAIL receptors have shown that TRAIL-R1 and TRAIL-R2 undergo clathrin dependent endocytosis upon ligand activation (Austin *et al.*, 2006; Kohlhaas *et al.*, 2007; Zhang *et al.*, 2008). In contrast to TNFR and FAS, internalization is not required for effective apoptotic signaling by TRAIL receptors (Austin *et al.*, 2006; Kohlhaas *et al.*, 2007). Interestingly, activation of TRAIL-R2 results in caspase dependent cleavage of clathrin and this attenuates internalization (Austin *et al.*, 2006). These studies further suggest that endocytosis negatively regulates apoptotic signaling and that blocking endocytosis (*e.g.*, by expression of a dominant negative dynamin mutant or by inhibition with chlorpromazine) potentiates TRAIL-induced apoptosis (Austin *et al.*, 2006; Zhang *et al.*, 2008). One study has found that the TRAIL receptors are localized predominantly in the cytosol in TRAIL-resistant breast cancer cells while they are localized on the plasma membrane on TRAIL-sensitive cells (Zhang and Zhang, 2008). Inhibition of clathrin mediated endocytosis increased the cell surface expression of TRAIL receptors and the sensitivity to TRAIL-mediated apoptosis in these resistant breast cancer cells (Zhang and Zhang, 2008). This suggests that a defect in proper trafficking of the TRAIL receptors could account for TRAIL resistance. No mechanism was described to account for the preferential localization of the TRAIL receptors in the cytosol of resistant cells.

V. OVERCOMING TRAIL RESISTANCE

Many studies in the literature have investigated the combination of a wide range of drugs with TRAIL in order to potentiate cell death and/or overcome resistance. This has also been investigated in breast cancer cells and, as will be outlined below, the results of many studies suggest that TRAIL may have the widest use in treating breast cancer when used in combination with other agents.

Combinations of chemotherapy with TRAIL have been extensively studied in many cancer cell types (Ashkenazi *et al.*, 2008). In breast cancer cells, the combination of TRAIL with chemotherapeutic drugs commonly used in the treatment of breast cancer can enhance the induction of apoptosis in the cancer cells (Buchsbaum *et al.*, 2003; Keane *et al.*, 1999; Singh *et al.*, 2003). A wide range of drugs has been tested in these studies including camptothecin, doxorubicin, etoposide, 5-fluorouracil, melphalan, methotrexate, paclitaxel, vincristin, and vinblastin. While each of the drugs can enhance TRAIL-mediated apoptosis in some of the breast cancer cells tested, the most consistent finding across the three studies is that doxorubicin synergistically enhances TRAIL-mediated apoptosis (Buchsbaum *et al.*, 2003; Keane *et al.*, 1999; Singh *et al.*, 2003). Importantly, the combination of TRAIL with chemotherapeutic drugs can overcome the intrinsic resistance to TRAIL in breast cancer cell lines (Buchsbaum *et al.*, 2003; Keane *et al.*, 1999; Singh *et al.*, 2003). Similarly, tumor xenograft studies using the TRAIL-sensitive MDA-MB-231 cell line have shown that the combination of TRAIL and chemotherapeutic drugs more effectively inhibits the growth of tumors than either alone (Buchsbaum *et al.*, 2003; Singh *et al.*, 2003). Several mechanisms have been proposed by which chemotherapeutic drugs enhance TRAIL-mediated apoptosis in the resistant breast cancer cells. In one study, concurrent administration of TRAIL and the chemotherapeutic agent caused markedly increased caspase activation. Interestingly, drugs

that themselves activated caspases interacted synergistically with TRAIL while those that did not activate caspases did not enhance TRAIL-mediated apoptosis (Keane *et al.*, 1999). Caspase inhibition using the pancaspase inhibitor ZVAD-FMK blocked the cell death induced by TRAIL, the caspase activating chemotherapeutic drugs, and the combination of the two. In this study, using simultaneous treatment with TRAIL and chemotherapeutic drugs, no consistent change in mRNA for TRAIL receptors or other apoptosis regulators was identified. This suggested that the independent activation of caspases by TRAIL and the chemotherapeutic drug accounted for the synergism. A second study found that a 24 hour pretreatment of breast cancer cells with chemotherapeutic drugs resulted in an up regulation of TRAIL-R1 and TRAIL-R2 mRNA and protein and that this correlated with the increased sensitivity of the cells to TRAIL (Singh *et al.*, 2003). This study found that simultaneous treatment of cells with TRAIL and chemotherapeutic drug or pretreatment with TRAIL followed by the chemotherapeutic drug was not as effective as preincubation of the cells with the chemotherapeutic drug. In addition, up regulation of the mRNA for proapoptotic Bcl2 family members (e.g., BAX and BAD) was observed in cells pretreated with chemotherapeutic drugs. Consistent with the first study, this second study also found that the chemotherapeutic drugs activated caspases and that caspase inhibition blocked the toxicity of the drugs, TRAIL, and the combination (Singh *et al.*, 2003). As in cell lines from other tumor types, the interaction of TRAIL and chemotherapeutic drugs appears independent of p53 status in the cell lines studied (Ashkenazi *et al.*, 2008; Keane *et al.*, 1999; Singh *et al.*, 2003). The ability of chemotherapeutic drugs to enhance TRAIL-mediated apoptosis and overcome the intrinsic resistance of breast cancer cells to TRAIL provides a rationale for combining TRAIL with chemotherapeutic drugs in clinical trials.

Radiation also enhances TRAIL-mediated apoptosis in cell lines and in tumor xenografts (Buchsbaum *et al.*, 2003; Chinnaiyan *et al.*, 2000). Mechanistic studies suggest that radiation results in up regulation of TRAIL-R2 in a p53 dependent fashion (Chinnaiyan *et al.*, 2000). Interestingly, TRAIL-R2 was independently identified as a p53 regulated gene induced by DNA damage (Wu *et al.*, 1997). However, while the combination of radiation and TRAIL appears to be synergistic, the utility of this approach to treating breast cancer is limited by the systemic nature of the disease.

While chemotherapeutic agents have shown promise in combination with TRAIL, they also may increase toxicity. For example, while we found that chemotherapeutic drugs could enhance TRAIL-mediated apoptosis in resistant breast cancer cell lines, these combinations also resulted in increased death of normal mammary epithelial cells (Keane *et al.*, 1999). This has led to an interest in combining TRAIL with targeted agents in breast cancer and in other tumor types in an effort to identify combinations that may potentiate the death of tumor cells without increasing toxicity (Ashkenazi *et al.*, 2008).

HER-2 is a member of the EGFR family that is amplified and overexpressed in 15–30% of breast and ovarian cancers (Slamon *et al.*, 1987; Slamon *et al.*, 1989; Tyson *et al.*, 1991; Zhang *et al.*, 1989). The majority of breast cancer cell lines with HER-2 amplification are resistant to TRAIL-mediated apoptosis (Buchsbaum *et al.*, 2003; Chinnaiyan *et al.*, 2000; Rahman *et al.*, 2009). The humanized anti-HER-2 antibody, trastuzumab (Herceptin®), has clinical activity alone and in combination with chemotherapy in metastatic breast cancer, but only when HER-2 is amplified (Pegram *et al.*, 1998; Pegram and Slamon, 1999; Slamon *et al.*, 2001; Vogel *et al.*, 2002). TRAIL-induced apoptosis could be enhanced in some resistant breast and ovarian cancer cell lines with HER-2 amplification (e.g., SKBR3, MDA-MB453, and SKOV3) when the cells were pretreated with trastuzumab (Cuello *et al.*, 2001; Dubska *et al.*, 2005). There was no interaction between TRAIL and trastuzumab in cells without HER-2 amplification. Mechanistic studies demonstrated that trastuzumab induces down-regulation of the HER-2 protein and that this results in inhibition of AKT kinase activity

(Cuello *et al.*, 2001). Also, AKT inhibition resulted in increased TRAIL sensitivity in these cells and expression of constitutively active AKT inhibited both TRAIL-mediated apoptosis and its potentiation by trastuzumab. In contrast to these results, investigation of another cell line (BT474) with HER-2 amplification showed that trastuzumab decreased TRAIL-mediated apoptosis (Dubska *et al.*, 2005). As in the report by Cuello *et al.*, this study found that incubating cells with trastuzumab resulted in decreased AKT kinase activity in the BT474 cells. However, in these cells this lead to a decrease in the surface expression of TRAIL-R1 and TRAIL-R2 and resistance to TRAIL-induced apoptosis. How a decrease in AKT activity can lead to such different results is unclear. Recent studies have demonstrated that different isoforms of AKT have different biological roles and different affects on apoptosis (Irie *et al.*, 2005; Kim *et al.*, 2009; Maroulakou *et al.*, 2007). Thus, it is possible that the opposing effects of trastuzumab on TRAIL-induced apoptosis are mediated by different AKT isoforms. Interestingly, the BT474 cell line expresses ER in addition to amplified HER-2 (Neve *et al.*, 2006; Rahman *et al.*, 2009). This result suggests that within HER-2 amplified breast cancers, there may be distinct subgroups that will have very different outcomes if TRAIL and trastuzumab are combined. Thus, tumors with HER-2 amplification may benefit from combined molecularly targeted therapies of TRAIL and trastuzumab. However, more studies will be needed to identify which tumors do and which do not benefit.

Epidermal growth factor receptor (EGFR) activity can attenuate death receptor mediated apoptosis and EGFR inhibition can increase sensitivity of cancer cells to TRAIL (Bremer *et al.*, 2005; Gibson *et al.*, 2002; Gibson *et al.*, 1999; Park and Seol, 2002; Shrader *et al.*, 2007; Teraishi *et al.*, 2005). High levels of EGFR expression are frequently seen in triple-negative/basal-like breast cancer cells (Korsching *et al.*, 2002; Livasy *et al.*, 2006; Neve *et al.*, 2006; Rahman *et al.*, 2009). EGFR inhibition can enhance TRAIL-mediated apoptosis in EGFR-expressing breast cancer cells that are already sensitive to TRAIL (*i.e.*, the mesenchymal triple-negative/basal-like breast cancer cell lines) but not in TRAIL-resistant cancer cell lines (Rahman *et al.*, 2009). This suggests that the expression of EGFR in breast cancer cells can modulate TRAIL sensitivity but that it is not the primary reason for resistance to TRAIL-mediated apoptosis. The mechanisms by which EGFR inhibition enhances TRAIL-induced apoptosis in breast cancer cells are not known, but studies in other tumor cell types implicate the inhibition of AKT in mediating these effects (Bremer *et al.*, 2005; Gibson *et al.*, 2002; Gibson *et al.*, 1999; Henson *et al.*, 2003; Shrader *et al.*, 2007; Teraishi *et al.*, 2005). A variety of possible mechanisms downstream of AKT inhibition have been suggested by these studies, including decreased cFLIP expression, decreased XIAP expression, inactivation of Bcl-xL, and decreased Mcl-1 expression. Overall, these data support the investigation of EGFR inhibitors in combination with TRAIL.

ER expressing cell lines have all been resistant to the induction of apoptosis by TRAIL alone (Buchsbaum *et al.*, 2003; Keane *et al.*, 1999; Singh *et al.*, 2003). These studies have shown synergistic interactions between TRAIL and chemotherapeutic drugs demonstrating that the TRAIL resistance can be overcome in ER positive cells. ER positive breast cancers are treated with agents that inhibit the activity of the ER including the selective ER modulators tamoxifen (Jordan and Brodie, 2007). A recent study demonstrated that tamoxifen can enhance TRAIL-induced apoptosis in breast cancer cell lines *in vitro* and *in vivo* (Lagadec *et al.*, 2008). Surprisingly, tamoxifen enhanced TRAIL-mediated apoptosis in both ER positive (MCF 7 and T47D) and ER negative (MDA-MB-231 and BT20) breast cancer cell lines. Tamoxifen has been shown to induce apoptosis in an ER independent fashion (Mandlekar and Kong, 2001). Possible mechanisms include activation of Jun N-terminal kinase signaling, activation of p38 signaling, induction of oxidative stress, and induction of ceramide production (Mandlekar and Kong, 2001). Further work is necessary to understand the enhancement of TRAIL-induced apoptosis by tamoxifen.

TRAIL receptors can activate the anti-apoptotic transcription factor NF- κ B (Chaudhary *et al.*, 1997; Degli-Esposti *et al.*, 1997a; Schneider *et al.*, 1997b). NF- κ B can protect cells from a variety of apoptotic stimuli by increasing expression of anti-apoptotic proteins (Baeuerle and Baltimore, 1996; Beg and Baltimore, 1996; Mayo *et al.*, 1997; Ravi *et al.*, 2001; Van Antwerp *et al.*, 1996; Wang *et al.*, 1998). Both TRAIL-sensitive and TRAIL-resistant breast cancer cells have detectable NF- κ B activity in nuclear extracts prior to treatment with TRAIL which increases upon TRAIL treatment (Keane *et al.*, 2000). In TRAIL-sensitive breast cancer cells, the activation of caspases occurs within minutes of ligand addition to the cells so that the induced NF- κ B activity is not likely to have time via transcriptional activation to inhibit the apoptosis (Keane *et al.*, 2000). In TRAIL-resistant breast cancer cell lines, inhibition of NF- κ B by overexpression of a genetic inhibitor increased TRAIL-mediated apoptosis (Keane *et al.*, 2000). Similar enhancement of TRAIL-mediated apoptosis has been seen in other tumor types (Jeremias and Debatin, 1998; Jeremias *et al.*, 1998). A recent paper, using the MDA-MB-435 cell line has demonstrated that aspirin can enhance TRAIL-mediated apoptosis *in vitro* and in xenografts (Lu *et al.*, 2008). They found that aspirin treatment resulted in proteasomal degradation of survivin and similarly that RNAi based down regulation of survivin enhanced TRAIL-mediated apoptosis (Lu *et al.*, 2008). Interestingly, salicylates have been shown to inhibit NF- κ B at the concentrations used in this study (Yin *et al.*, 1998). However, the study by Lu *et al.* did not investigate whether inhibition of NF- κ B contributed to the enhancement of TRAIL-mediated apoptosis (Lu *et al.*, 2008). One caveat in this work is that while the MDA-MB-435 cell line was originally derived from a patient with breast cancer, there is controversy in the literature as to whether the cell line in use by investigators originated from a breast cancer or melanoma (Chambers, 2009; Lacroix, 2009). None-the-less, together these results suggest that small molecule inhibitors of NF- κ B could be a means to enhance TRAIL-mediated apoptosis in breast cancer cells.

Histone deacetylase (HDAC) inhibitors are currently under investigation for the treatment of cancer (Carew *et al.*, 2008). HDACs mediate deacetylation of histones, generally leading to chromatin compaction in histones and transcriptional repression. By altering the epigenetic regulation of gene transcription, HDAC inhibitors have been shown to induce cell-cycle arrest, promote differentiation, and cause tumor cell death (Carew *et al.*, 2008). One HDAC inhibitor, vorinostat, has been approved for the treatment of cutaneous T-cell lymphoma (Duvic and Vu, 2007). Preclinical studies combining HDAC inhibitors with TRAIL have shown synergistic induction of apoptosis in tumor cells (Fulda, 2008). Multiple mechanisms have been demonstrated for the enhancement of TRAIL in tumor cells by HDAC inhibitors including up regulation of TRAIL receptors, redistribution of TRAIL receptors to membrane lipid rafts, increased activation of the mitochondrial pathway, down-regulation of antiapoptotic proteins (*e.g.*, cFLIP, antiapoptotic Bcl-2 family members, and survivin), and up regulation of proapoptotic Bcl-2 family members (Fulda, 2008). In breast cancer cells, several HDAC inhibitors have been shown to enhance TRAIL-mediated apoptosis (Chopin *et al.*, 2004; Singh *et al.*, 2005). One study found that the synergistic enhancement of TRAIL-mediated apoptosis in several TRAIL-resistant cell lines by HDAC inhibition was secondary to p21 expression (Chopin *et al.*, 2004). The second study described enhanced TRAIL-mediated apoptosis in both TRAIL-sensitive and resistant cell lines by HDAC inhibitors (Singh *et al.*, 2005). This study found that HDAC inhibition resulted in up regulation of TRAIL-R1, TRAIL-R2, and proapoptotic Bcl-2 family members. Interestingly, the up regulation of TRAIL-R1 and TRAIL-R2 by HDAC inhibitors is mediated by NF- κ B (Shetty *et al.*, 2005; Singh *et al.*, 2005). This latter result suggests that NF- κ B inhibition (described above) may either enhance or inhibit TRAIL-mediated apoptosis depending on the context.

Triterpenoids are naturally occurring compounds synthesized by many plants and two of the naturally occurring triterpenoids, oleanolic acid and ursolic acid, have weak anti-inflammatory and anti-tumor effects *in vivo* (Liby *et al.*, 2007). More potent synthetic derivatives 2-cyano-3,12-dioxooleana-1,9(11)-diene-28-oic acid (CDDO) and its derivative 1-(2-cyano-3,12-dioxooleana-1,9(11)-diene-28-oyl) imidazole (CDDO-Im) have been shown to enhance TRAIL-mediated apoptosis in TRAIL-resistant breast cancer cells both *in vitro* and *in vivo* (Hyer *et al.*, 2005). These studies found that CDDO and CDDO-Im enhance apoptosis induced by TRAIL and by agonistic antibodies to either TRAIL-R1 or TRAIL-R2. These studies further showed that CDDO and CDDO-Im down-regulate the expression of the antiapoptotic protein cFLIP and upregulate the mRNA and protein expression of TRAIL-R1 and TRAIL-R2. The mechanism by which these compounds regulate the expression of these proteins is unclear (Hyer *et al.*, 2005).

VI. FUTURE DIRECTIONS

The data discussed above provide evidence that TRAIL or agonist antibodies directed at TRAIL-R2 could have clinical activity in the treatment of breast cancer. The underlying mechanisms that control TRAIL sensitivity in breast cancer cells have not been clearly defined. The phenotypic markers such as triple-negative/basal-like features and mesenchymal gene expression (*e.g.*, vimentin) may act as surrogate biomarkers to predict the patients most likely to benefit from TRAIL treatment. Clinical trials aimed at these patients would be a logical first step in the clinic. However, further work is necessary to identify the true determinants of TRAIL sensitivity or resistance in breast cancer cells as these are more likely to be robust biomarkers. In addition, a large body of evidence suggests that resistance to TRAIL may be overcome in the other types of breast cancer by combinations of TRAIL ligands with various agents including chemotherapy and targeted therapies. Again, understanding the underlying molecular mechanisms that determine resistance may ultimately lead to more efficacious agents to combine with TRAIL in clinical studies.

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Fig. 1. Apoptosis pathways. Different stimuli and cell stresses result in activation of distinct initiator caspases (*i.e.*, caspases 2, 8, 9, 10, and 12 shown in gray boxes) as discussed in the text. These in turn cleave and activate down stream effector caspases (*e.g.*, caspases 3, 6 and 7). The primary initiator caspase can activate secondary initiator caspases as discussed in text (dashed arrows). The activation of effector caspases leads to apoptotic cell death.

Fig. 2.

Death receptor pathway. TNF family death ligands (e.g., TNF, FAS, TRAIL) bind to their cognate receptors and initiate the formation of the death inducing signaling complex (DISC). All of the TNF family receptors which induce apoptosis contain a highly conserved death domain (DD) in their cytoplasmic tails. The adaptor protein FADD contains an N-terminal death effector domain (DED) and a C-terminal DD. FADD is recruited to the activated receptor by homotypic interactions between the C-terminal DD of FADD and the DD of the receptor. Inactive caspase 8 and caspase 10 zymogens are recruited to the DISC by homotypic interactions between the N-terminal DED domains of the caspases and FADD. cFLIP can be recruited to the DISC and prevents recruitment of caspase 8 or 10. The recruitment of caspase 8 or caspase 10 to the DISC results in activation of the caspases and auto-processing into the active forms of the caspase (reviewed in Riedl and Shi, 2004). Activated caspase 8 or caspase 10 can directly activate effector caspases (e.g., caspases 3, 6, and 7). Activated caspase 8 or caspase 10 also can cleave the BH3 only protein Bid. Cleaved Bid (tBid) translocates to the mitochondria where it activates the extrinsic pathway.

Fig. 3.

TRAIL receptors. TRAIL-R1 and TRAIL-R2 are type I transmembrane proteins that contain a DD. Ligand binding to these receptors results in activation of caspases. TRAIL-R3 is a glycosphospholipid-anchored cell surface protein, TRAIL-R4 is a transmembrane protein lacking an intact DD, and TRAIL-R5 is a secreted protein. These proteins bind TRAIL but are unable to activate caspases. These receptors act as decoy receptors and can inhibit TRAIL-mediated apoptosis by competing with TRAIL-R1 and TRAIL-R2 for the ligand.

Fig. 4. TRAIL agonists. Clinical trials testing TRAIL (a.k.a. rhApo2L), an agonistic TRAIL-R1 antibody (mapatumumab), and agonistic TRAIL-R2 antibodies (lexatumumab and apomab) are currently ongoing.

Fig. 5. TRAIL selectively kills mesenchymal triple-negative breast cancer cell lines. (Top) Growth inhibition of breast cancer cells incubated with TRAIL. Black lines represent mesenchymal triple-negative cell lines, green lines represent epithelial triple-negative cell lines; blue lines represent HER-2 amplified cell lines, and red lines represent ER positive cell lines. (Bottom) Characterization of ER and HER-2 expression in the breast cell lines. This figure is reproduced with kind permission of Springer Science and Business Media from Fig. 1 in (Rahman *et al.*, 2009).

Fig. 6.

Cell surface expression of TRAIL-R1 and TRAIL-R2 do not correlate with TRAIL-sensitivity. Mean cell surface expression of TRAIL-R1 and TRAIL-R2 in seven TRAIL-sensitive and seven TRAIL-resistant breast cancer cells was measured by flow cytometry (see text for discussion).