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Investigating TRAIL Sensitivity in Platinum-Resistant Ovarian Cancer

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Investigating TRAIL Sensitivity in Platinum-Resistant Ovarian Cancer

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Abstract

      Ovarian cancer is the deadliest gynecologic malignancy in the United States. While these tumors may have a promising initial response to platinum-based chemotherapies, the patient's prognosis is commonly hindered by the development of platinum resistant cancer. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been implicated as a potential treatment for many cancers based on its tumor selective nature. Combination therapies with TRAIL and platinum drugs have been shown to increase apoptosis and have been used in a variety of clinical trials, which have thus far failed to pass phase II.[1] Researchers have not yet elucidated the complex mechanism(s) of TRAIL sensitivity. The objective of the following experiments was to investigate TRAIL sensitivity in paired isogenic platinum resistant and sensitive ovarian cancer cell lines. TRAIL induced cell death quantification, death receptor quantification, and protein analysis was carried out. While all platinum resistant cell lines (A2780 CP200, IGROV1 CP, PEO4, and ABTR2) were sensitive to TRAIL, their isogenic platinum sensitive counterparts (A2780, IGROV1, and PEO1) are much less sensitive. In an effort to elucidate the mechanism of sensitivity in the platinum-resistant lines, known mediators of the TRAIL induced killing pathway were quantified. An increase in DR4 and DR5 receptors was observed in PEO1, PEO4, and ABTR2 cell lines. An increase in FADD was observed at the protein level in A2780 CP200 and IGROV1 CP cell lines. However, FADD knockdown in IGROV1 CP cells failed to protect against TRAIL induced cell death. IGROV1 CP cells also display a similar tolerance for tBID and an increase in native caspase-8 levels in comparison to IGROV1 parental cells. BID knockdown in IGROV1 CP cells only provided minimal protection to TRAIL treatment. TRAIL sensitivity is correlated with platinum resistance in three sets of isogenic ovarian cancer cell lines. TRAIL treatment should be considered for ovarian cancer patients who have experienced relapse after platinum-based chemotherapy. Future experiments should further investigate the role FADD may play in TRAIL sensitivity, c-FLIP levels, TRAIL activating the NF-kB pathway, and the potential role of TRAIL decoy receptors in TRAIL sensitivity. TRAIL is a promising treatment for ovarian cancer.

Introduction

Ovarian cancer is the most lethal gynecologic malignancy in the United States and accounts for approximately 14,600 deaths annually.[2,3] Primary treatment includes the use of platinum-based chemotherapeutic agents in conjunction with surgical removal, if possible.[3] Secondary treatment may include the use of poly-ADP ribose polymerase (PARP) inhibitors, which are a new targeted therapy. While the initial response to platinum treatment may be good with an average progression-free survival time of 18 months, 80% of patients experience relapse/progression largely in part due to platinum resistance.[2,4,5] While the exact mechanisms of platinum resistance remain unknown four common tenets have been elucidated: 1) decreased intracellular drug accumulation, 2) drug inactivation via thiol-containing proteins, 3) increased DNA repair or DNA damage tolerance, and 4) cell cycle checkpoint mutations and anti-apoptotic mechanisms.[2,6] Platinum resistance remains a major obstacle in ovarian cancer treatment.

A potential treatment for ovarian cancer is tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). TRAIL has been used in animal models and *in vitro* to kill non-small cell lung cancer, colon cancer, breast cancer, renal cancer, and glioblastoma.[7] TRAIL primarily targets transformed and malignant cells causing few side effects in normal tissues, which makes TRAIL an ideal anti-cancer agent.[8] The mechanism behind the tumor selectivity of TRAIL is not well understood. Membrane bound TRAIL is naturally produced by natural killer cells, B cells, monocytes, and dendritic cells as part of the human immune response.[9] Advanced stage ovarian cancer patients who survived over 5 years post-diagnosis had TRAIL expression levels 2.2-fold higher than advanced stage ovarian cancer patients who survived less than one-year post-diagnosis.[8] When used as a treatment, TRAIL is modified to just possess the extracellular receptor-binding domain, which is soluble without the other membrane bound domains.[7] While studies have shown that ovarian cancer cell lines such as IGROV1 respond well to TRAIL treatment (72 hours), others (A2780, SKOV-3) require combination therapies to induce apoptosis.[10] One obstacle for TRAIL treatment is that TRAIL has a very low retention time in the body with studies suggesting just 30 minutes.[7] To overcome the short half-life, researchers have developed modified TRAIL containing noncovalent tags (promote oligomerization/stability), modified TRAIL containing covalent linkages to molecules with a long half-life (human serum albumin), monoclonal antibodies to specific TRAIL receptors (ant-DR4, anti-DR5), and nanoparticle complex systems for sustained delivery.[7] Viral vectors such as adenoviruses, lentiviruses, and oncolytic herpes simplex virus-1 have been successfully used to deliver secretable TRAIL cDNA to cancer cells.[7] Human mesenchymal stem cells genetically modified to secrete TRAIL have been grafted near tumors and have been used to treat glioblastoma, renal cell carcinoma, malignant fibrous histiocytoma, and lung cancers in animal models.[7] While TRAIL treatment technologies have been successful in animal models, clinical trials using these methods in combination with other chemotherapeutics have failed to pass the Phase II stage.[7] Ovarian cancer has only been a target of one clinical trial using TRAIL (in combination with paclitaxel and carboplatin), which was

discontinued after Phase II.[11] Discovering the mechanisms underlying TRAIL sensitivity prior to clinical trials should improve the efficacy of TRAIL treatment.

TRAIL initiates apoptosis after binding to either TRAIL receptor DR4 or DR5 (Figure 1).[12] The relative amount of each TRAIL receptor may be important as three decoy receptors (DcR1, DcR2, osteoprotegerin) compete for and bind TRAIL, yet lack the intracellular death domain to initiate cell death.[12] After TRAIL binding, the ligand-receptor complex forms a death-inducing signaling complex (DISC) by recruiting Fas-associated death domain (FADD) protein to the death domain of the receptor complex.[7,13] FADD binds to procaspase-8/10 completing the DISC.[14] Autocatalytic activity between procaspases releases active caspase-8/10 into the cytoplasm.[14] Caspase-8/10 cleaves and activates effector caspases such as caspase-3, which induces mass proteolysis leading to apoptosis.[7,14] The direct cleavage of caspase-3 by caspase-8 is referred to as the extrinsic death pathway. Type I cells are cells that get sufficient caspase-8/10 cleavage to initiate apoptosis by means of the extrinsic pathway alone.[13,14] Type II cells require further caspase activation by the intrinsic pathway (mitochondrial death pathway) in addition to the extrinsic pathway to undergo apoptosis.[14] In the case of TRAIL-induced apoptosis, type II cell death requires BID cleavage by caspase-8/10, which releases truncated BID (tBID).[13,14] Activation of the intrinsic pathway by tBID promotes mitochondrial permeablization through Bax/Bak pore formation, which prompts cytochrome c release into the cytosol.[13,14] Cytochrome c binds apoptotic protease activating factor 1 (APAF-1) inducing the formation of the apoptosome through procaspase-9 binding.[14] Procaspase-9 cleavage occurs now forming the active initiator caspase-9, which activates effector caspases that cause apoptosis.[7,14] Research has suggested that tBID formation regulates TRAIL-induced apoptosis in type II cells.[13] Epithelial ovarian cancer is suspected to undergo type II cell death.[14] A common technique for quantifying cell death is flow cytometry in conjunction with Annexin V/propidium iodide (PI) staining. Annexin V binds to translocated phosphatidylserine on the cell surface of early and late apoptotic cells. PI binds to nuclear DNA of cells that are in either late stage apoptosis or necrosis. In conjunction, Annexin V/PI staining helps identify how cells were killed after a given treatment.

A host of proteins regulate the processes of type I and type II cell death. Initiator caspases (8/10) compete with c-FLIP ((FADD-like IL-1β-converting enzyme)-inhibitory protein) for FADD binding.[13,14] Inhibitor of apoptosis proteins (IAPs) such as XIAP bind and inhibit effector caspases, while other IAPs are inhibited by SMAC/DIABLO released during mitochondrial membrane permeablization.[13] The intrinsic pathway is positively regulated by pro-apoptotic proteins Bax, Bak, and BID and negatively regulated by anti-apoptotic proteins Bcl-2, Bcl-XL, and Mcl-1.[13,14] The expression level of these regulators ultimately dictates if sufficient effector caspases (3/7) are activated to initiate TRAIL-induced apoptosis.
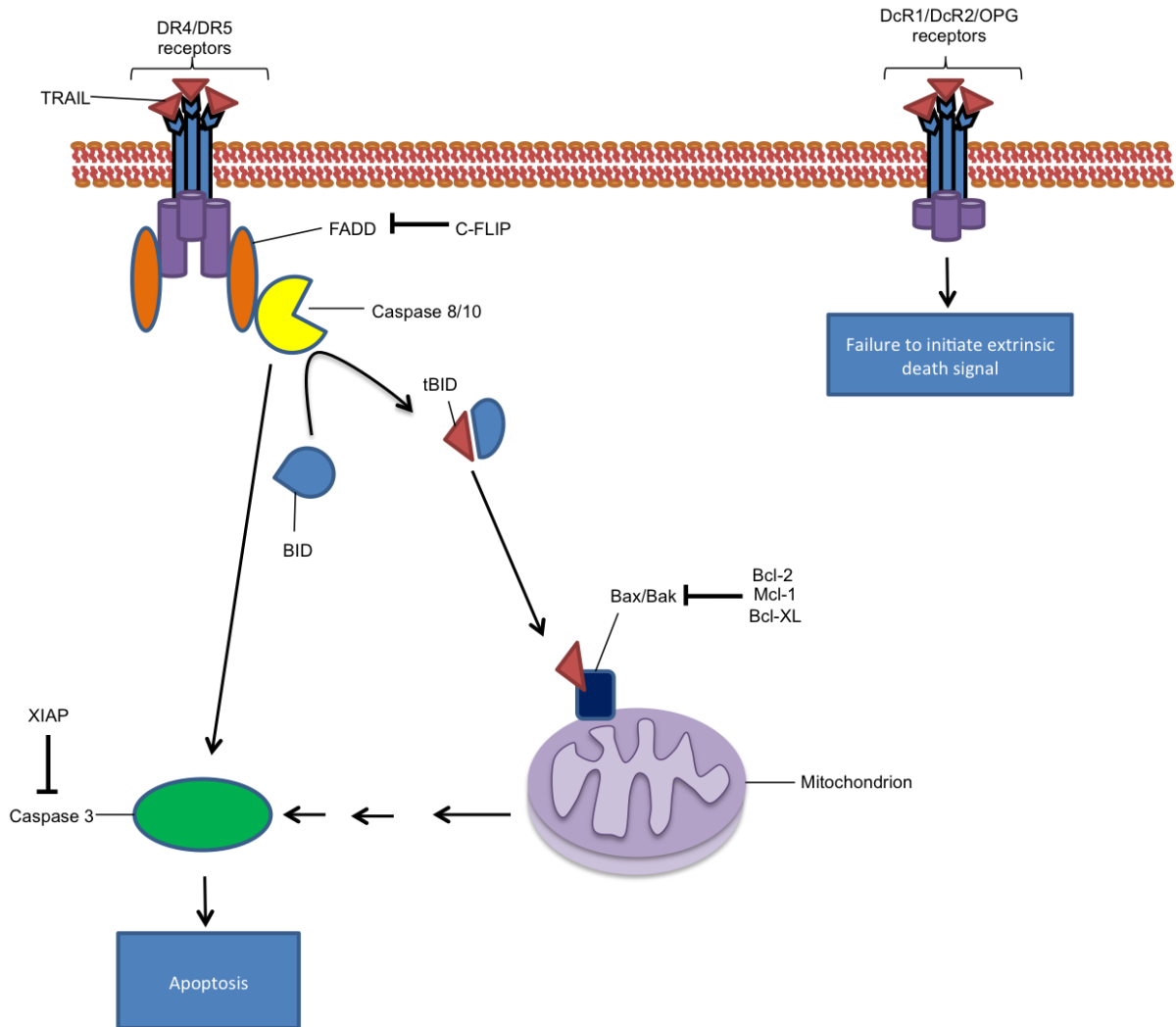
Figure 1. TRAIL Pathway. TRAIL binds to extrinsic death receptors DR4/DR5 initiating receptor oligomerization further recruiting FADD. Upon the activation of caspase-8, the pathway can proceed by directly cleaving caspase-3 and/or by cleaving BID, which initiates the intrinsic pathway. Most cells rely on both the direct cleavage of caspase-3 and tBID for sufficient caspase-3 cleavage and apoptosis. Decoy receptors, DcR1 and DcR2, lack an internal death domain and do not initiate cell death.

TRAIL resistance in several ovarian cancer cell lines has been overcome using TRAIL in combination with cytotoxic agents (cisplatin, etc.), peroxisome proliferator-activated receptor γ (PPARγ), and proteasome inhibitors.[15-17] The mechanisms that mediate TRAIL sensitivity and resistance are not well understood. Preliminary data gathered by the Kaufmann lab suggested that platinum-resistant ovarian cancer cell lines might be more sensitive to TRAIL than platinum-sensitive cells. The objective of the following experiments was to determine TRAIL sensitivity status in several platinum-sensitive/platinum-resistant isogenic ovarian cancer cell lines and

investigate possible mechanisms that may contribute to TRAIL sensitivity. The cell lines investigated included: platinum-sensitive (IGROV1, A2780, PEO1), platinum-resistant (IGROV1 CP, A2780 CP200, PEO4), and PARP inhibitor resistant platinum-resistant (ABTR2) (Table 1).

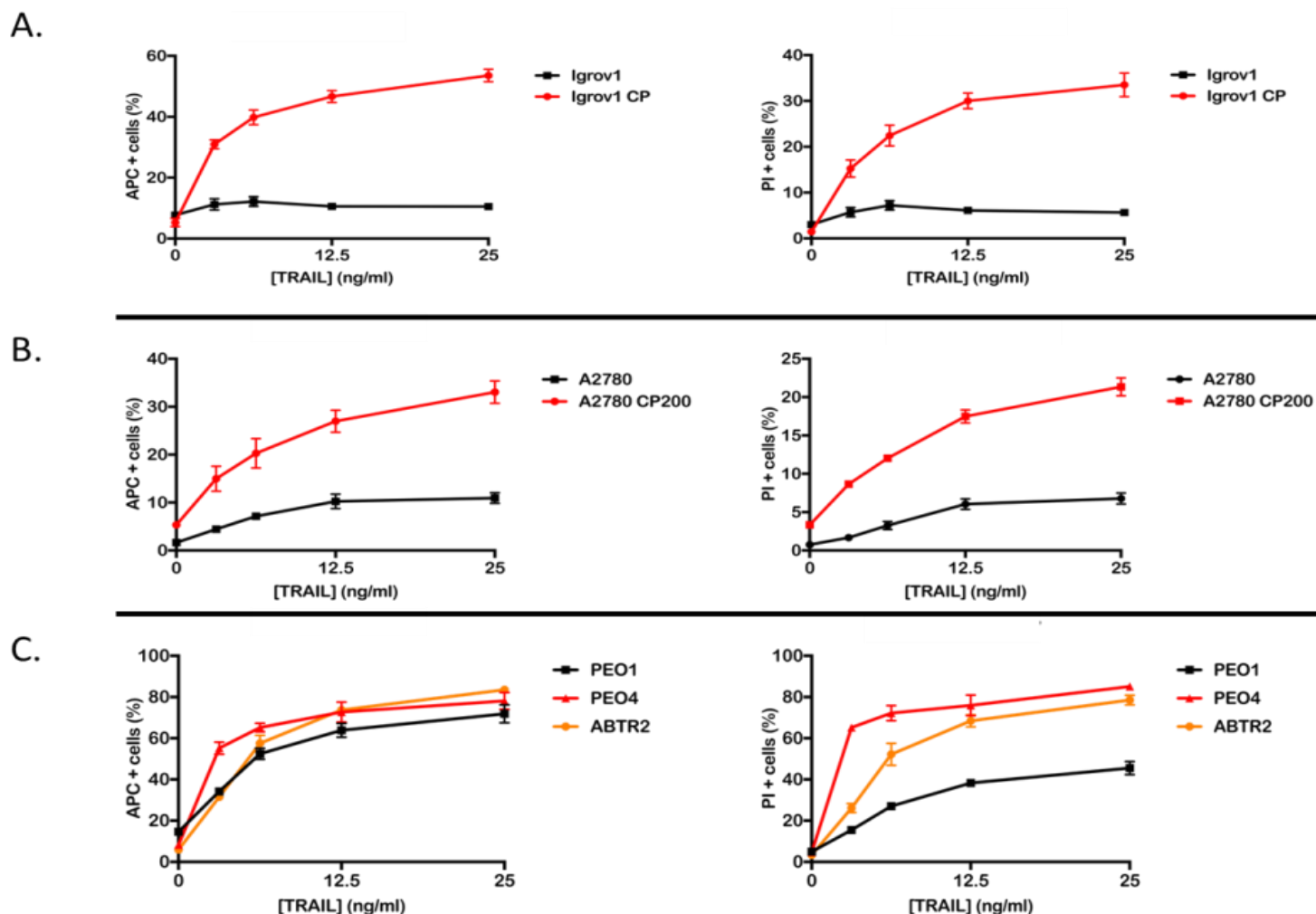**Table 1. Ovarian cancer cell line platinum status, histological subtype, and mutation status.**

Cell line	Platinum status	Subtype	Mutations
IGROV1	Sensitive	Endometriod	TP53, BRCA1/2
IGROV1 CP	Resistant	Endometriod	TP53, BRCA1/2
A2780	Sensitive	Endometriod	BRCA2
A2780 CP200	Resistant	Endometriod	N/A
PEO1	Sensitive	High-grade serous	TP53, BRCA2
PEO4	Resistant	High-grade serous	TP53, BRCA2
ABTR2*	Resistant	High-grade serous	TP53, BRCA2

*PEO1 cell line made cross-resistant to PARP inhibitors and platinum-based drugs

Results

Platinum-resistant cell lines are more sensitive to recombinant TRAIL treatment than their paired isogenic cell lines. Three sets of isogenic serous/adenocarcinoma ovarian cancer cell lines were treated with recombinant human TRAIL (3.125 ng/mL, 6.250 ng/mL, 12.500 ng/mL, 25.000 ng/mL) for 24-hours (Figure 2). Annexin V(APC+) was used to label early and apoptotic cells while propidium iodide (PI+) was used to label late apoptotic and necrotic cells. A higher percentage of cells that are positive for either label represents more death occurred as the result of the TRAIL treatment. An increased sensitivity to TRAIL is represented by an increase in the percentage of APC+ and/or PI+ cells between isogenic cell lines. All of the platinum resistant cell lines (IGROV1 CP, A2780 CP200, ABTR2, PEO4) showed increased sensitivity to TRAIL. Of the three sets of isogenic cell lines treated with a TRAIL concentration of 25 ng/mL, IGROV1 CP cells had the largest difference in apoptotic cells (+43%) compared to its isogenic pair IGROV1 (Figure 2A). A2780 CP200 cells demonstrated a modest difference in apoptotic cells (+22%) (Figure 2B). Interestingly, PEO1 cells underwent a large amount of apoptosis (71%) after 24-hours while PEO4 and ABTR2 cells demonstrated slightly higher percentages of 78% and 83% respectively (Figure 2C). PI labeling for both IGROV1/IGROV1 CP and A2780/A2780 CP200 cell line pairs followed similar trends to their APC labeled results (Figure 2A & 2B). PEO4 and ABTR2 cells were more PI+ showing that they are more sensitive to TRAIL than their parental cell line, PEO1 (Figure 2C). Cell lines PEO1, PEO4, and ABTR2 showed the greatest response to TRAIL treatment while the PEO1 was weaker as indicated by fewer late apoptotic and necrotic cells (Figure 2C). The experiment was performed in triplicate, and error bars represent the standard error of the mean. The y-axis for each isogenic pair is scaled differently.

Figure 2. Platinum-resistant cells are sensitive to TRAIL. Isogenic ovarian cancer cell lines underwent 24-hour treatment with TRAIL with concentrations up to 25 ng/mL. (A) IGROV CP, (B) A2780 CP200, (C) PEO4 and ABTR2 cells experienced a significant increase in cell death after TRAIL treatment in comparison with their platinum-sensitive parental cell line. The y-axis is scaled differently for each isogenic line. APC+ cells are either early or late apoptotic cells while PI+ cells are either necrotic or late apoptotic cells. A similar trend can be seen between APC+ and PI+ cells in all seven ovarian cancer cell lines. (n=3, error bars represent the standard error of the mean).

TRAIL sensitivity is not the result of an increased quantity of TRAIL death receptors on the surface of platinum-resistant cell lines. TRAIL death receptors DR4 and DR5, along with extrinsic death receptor FasR, were quantified using APC conjugated antibodies and flow cytometer techniques. FasR was quantified to analyze potential trends in extrinsic death receptor quantities and follows a similar pathway to TRAIL associated pathways. The experiment was performed in triplicate, and receptor expression levels were normalized to their respective control cell populations that were not exposed to antibodies. As shown in Figure 3, IGROV1 and IGROV1 CP cells had similar expression levels of DR4 and DR5 (n=3, error bars

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represent the standard error of the mean). A2780 and A2780 CP200 cells also followed the same trend with both expressing similar amounts of DR4 and DR5 (Figure 3). The PEO1 isogenic cell lines do not follow this trend. Both PEO4 and ABTR2 cell lines expressed higher levels of DR4 and DR5 than PEO1 cells (Figure 3). While the difference in protein expression is less than two-fold further experiments should be performed to analyze if increased TRAIL receptor expression in PEO4 and ABTR2 cells is the reason for their increased TRAIL sensitivity.
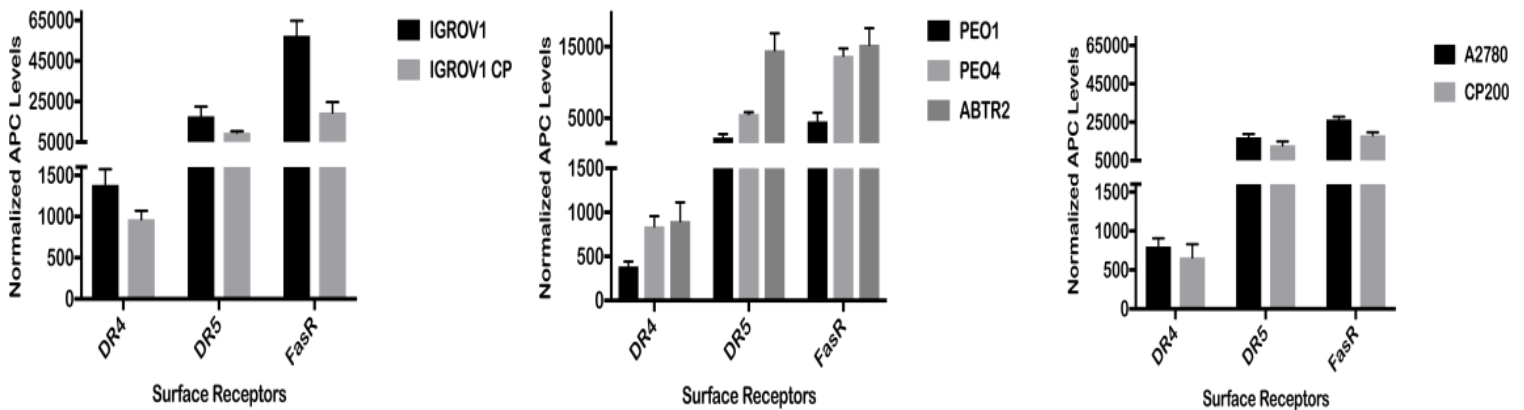
Figure 3. Surface Receptor Quantification. The relative quantity of TRAIL receptors (DR4/DR5) and extrinsic death receptor FasR were compared in isogenic platinum-sensitive (IGROV1/PEO1/A2780) and platinum-resistant (IGROV1 CP/PEO4/ABTR2/A2780 CP200) sets of ovarian cancer cell lines. (n=3, error bars represent the standard error of the mean).

FADD protein levels were upregulated in IGROV1 CP and A2780 CP200 cell lines while differential expression levels were found for other TRAIL pathway proteins. Native levels of important proteins in the TRAIL pathway were quantified using standard cell harvesting and western blotting protocols after 24-hour treatment with recombinant TRAIL. Protein analysis was performed for IGROV1, IGROV1 CP, A2780, and A2780 CP200 cell lines (experiment not yet successful for PEO1, PEO4, and ABTR2 cell lines). The selected proteins analyzed were caspase-8, XIAP, FADD, and BID. As shown in Figure 4, FADD expression was increased in the platinum-resistant cell lines IGROV1 CP and A2780 CP200 compared to their platinum-sensitive controls IGROV1 and A2780 (n=1). A similar trend was observed with XIAP protein expression levels yet to a lesser extent (Figure 4). Pro-apoptotic protein caspase-8 was increased in IGROV CP cells but not in A2780 CP200 cells (Figure 4). Opposing trends were observed for BID expression as A2780 CP200 cells had upregulated BID expression while IGROV1 CP cells had downregulated BID expression (Figure 4). The differential expression shown by caspase-8 and BID suggest there may be cell line specific differences that influence TRAIL sensitivity.

FADD is located at a critical location in the TRAIL pathway, and the trend observed in Figure 4 suggests FADD may play an important role in TRAIL sensitivity.
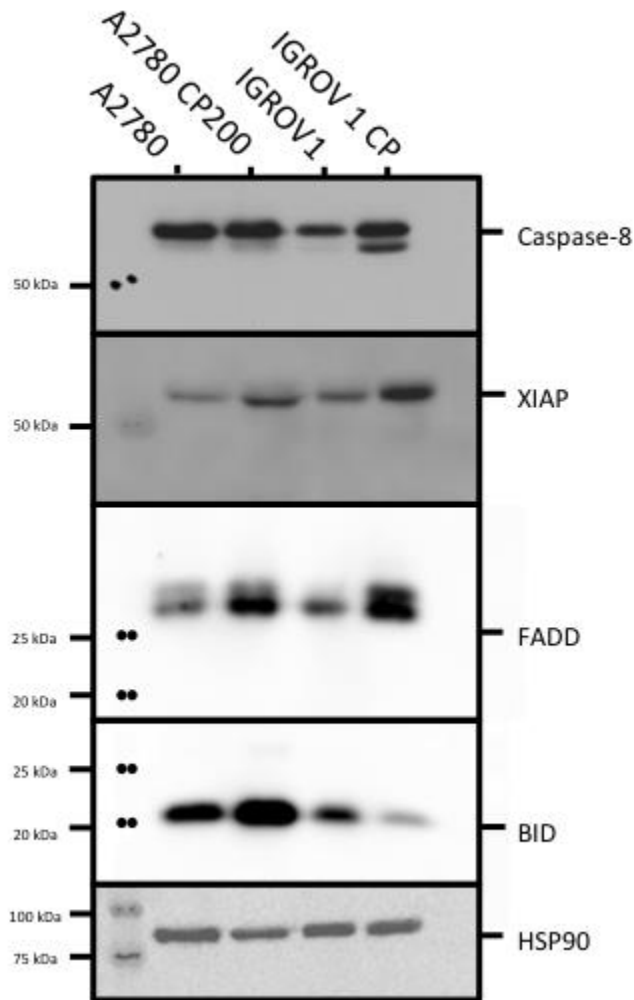
Figure 4. TRAIL pathway protein quantification and analysis. Important TRAIL pathway proteins caspase-8, XIAP, FADD, and BID were quantified in 2 isogenic cell line pairs (A2780/A2780 CP200, IGROV1/IGROV1 CP). The increase of FADD in both platinum-resistant isogenic cell lines is a target of interest. Protein levels are indicative of native levels. (n=1)

FADD mRNA levels were slightly increased in IGROV1 CP, PEO4, and A2780 CP200 cell lines. Untreated IGROV1, IGROV1 CP, PEO1, ABTR2, PEO4, A2780, and A2780 CP200 cells were harvested, and mRNA was extracted using a Qiagen RNeasy extraction kit. Quantitative PCR (qPCR) was performed, and mRNA values for platinum-resistant cell lines were normalized to their respective platinum-sensitive isogenic cell line. The resulting value provided the fold change difference of FADD mRNA from the control. As shown in Figure 5, IGROV1 CP cells had the greatest fold change increase of 2. ABTR2 cells expressed no fold change in FADD mRNA levels

(Figure 5). While FADD mRNA levels may be slightly greater in platinum-resistant cell lines it likely does not account for all of the increase in FADD protein levels seen in Figure 4.
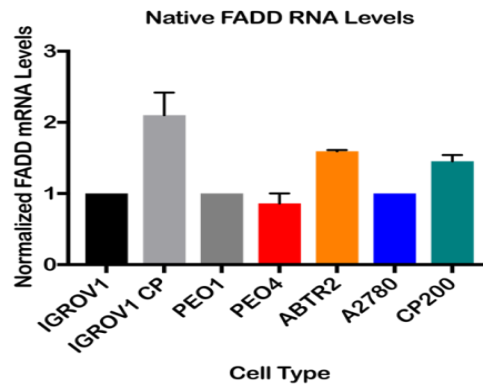
Figure 5. FADD mRNA levels in IGROV1, IGROV1 CP, PEO1, PEO4, ABTR2, A2780, and A2780 CP200 ovarian cancer cell lines. The values were normalized to the mRNA level of the platinum-sensitive cell line in each isogenic set. Data was collected via qPCR after RNA extraction. (n=2, error bars represent standard error of the mean).

Knockdown of FADD in platinum-resistant cell lines (IGROV1 CP, PEO4, ABTR2) using siFADD failed to decrease TRAIL sensitivity. FADD was knocked down to analyze if FADD mediated TRAIL sensitivity in platinum-resistant cell lines. Two different siFADD targeting different regions of FADD mRNA were used to silence FADD expression. In the IGROV1 and IGROV1 CP cell lines, siFADD#1 failed to decrease FADD mRNA (Figure 6A). Both siFADD#1 and #2 were successful at decreasing FADD mRNA and protein expression levels in the remainder of the cell lines (Figure 6A). The FADD mRNA levels were normalized to respective native mRNA levels and the platinum-sensitive isogenic cell line of each set. TRAIL sensitivity was assessed following the same procedure as shown in Figure 2. Shown in Figure 6B and 6C, FADD knockdown failed to decrease the sensitivity to TRAIL treatment in the platinum-resistant cell lines.
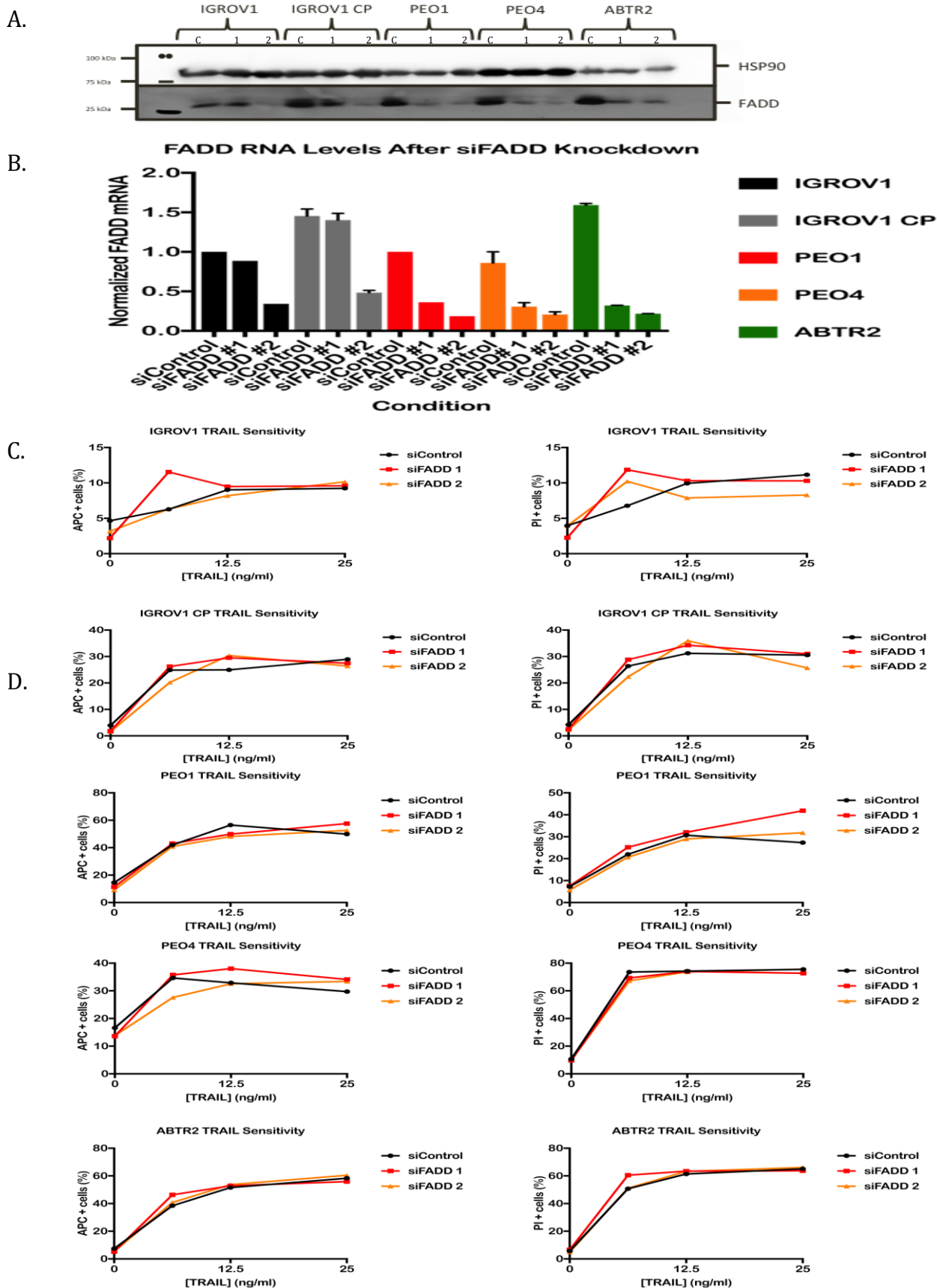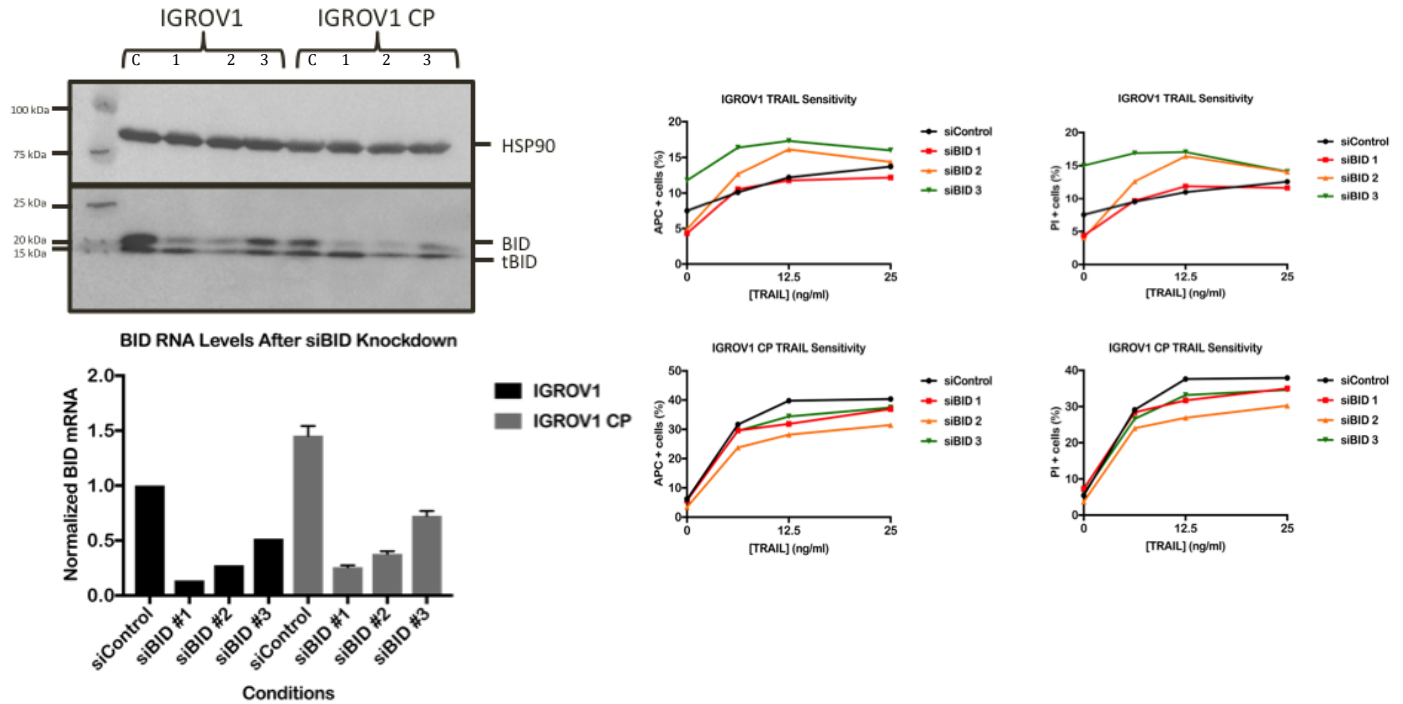
Figure 6. TRAIL sensitivity after FADD knockdown in IGROV1, IGROV1 CP, PEO1, PEO4,

and ABTR2 cell lines. Two different siFADD RNAs were used to knockdown FADD. (A) Protein levels of FADD after knockdown were collected 48 hours after transfection. HSP90 was used as a control for the protein levels while an empty vector plasmid (siControl) was used as the control for the overall knockdown for each cell line. Knockdown decreased protein levels except when siFADD#1 was used in the IGROV1 and IGROV1 CP cell lines. (B) qPCR data shows FADD mRNA levels after knockdown. Similar trends were observed between protein and mRNA levels. FADD mRNA levels were normalized to their respective cell line siControl and native mRNA levels. Protein lanes correlate with the labeled mRNA columns. (C) TRAIL sensitivity in IGROV1 and IGROV1 CP cell lines was tested using the same procedure used initially. (D) TRAIL sensitivity in PEO1, PEO4, and ABTR2 cell lines was tested using the same procedure used initially. APC+ cells are in early/late stage apoptosis while PI+ cells have undergone necrosis or are in late stage apoptosis. (n=1)

Knockdown of BID in platinum-resistant IGROV1 CP decreased TRAIL sensitivity. BID was knocked down in order to investigate the extent TRAIL sensitivity was reliant on the intrinsic death pathway. Three different siBID targeting different regions of BID mRNA were used to silence BID expression. All three siBID were successful at decreasing BID protein (Figure 7A) and mRNA levels (Figure 7B). Protein quantification showed that varying amounts of tBID were present at the time of treatment (Figure 7A). Knockdown of BID decreased TRAIL sensitivity for each of the siBID used (Figure 7C). The most drastic decrease was seen using siBID#2, which produced a decrease in the percentage of APC+ cells of nine percent and a decrease in PI+ cells of eight percent (Figure 7C).

IGROV1 CP cells do not show hypersensitivity to tBID. While IGROV1 CP cells have lower native BID levels than IGROV1 cells (Figure 4) they may possess a hypersensitivity to tBID due to their genetic makeup. In order to measure the amount of tBID IGROV1 and IGROV1 CP cells could tolerate without undergoing processes of cell death, tBID plasmid vectors were transfected into the cells using electroporation. The protein plasmid produced was conjugated with GFP. A separate set of cells were transfected with Bax-GFP producing plasmids as a control. Two days after transfecting the cells flow cytometry using Annexin V (APC+) was performed to identify the living cell population of each modified cell line. An arbitrary pre-determined survival threshold was set at the amount of a given protein 98% of the living population could withstand. GFP fluorescence at this survival threshold represents the maximum relative quantity of protein the cell can survive. A greater amount of GFP signal represents a higher tolerance for the tagged protein. If IGROV1 CP cells were hypersensitive to tBID, they would have less GFP signal at the survival threshold. As shown in Figure 8, this is not the case. IGROV1 CP cells tolerate tBID to the same extent as IGROV1 cells.

Figure 7. TRAIL sensitivity after BID knockdown in IGROV1 and IGROV1 CP cell lines. Three different siBID RNAs were used to knockdown BID. (A) Protein levels of BID after knockdown were collected 48 hours after transfection. HSP90 was used as a control for the protein levels while an empty vector plasmid (siControl) was used as the control for the overall knockdown for each cell line. Knockdown decreased protein and mRNA levels. (B) FADD mRNA levels were normalized to their respective cell line siControl and native mRNA levels. (C) TRAIL sensitivity in IGROV1 and IGROV1 CP cell lines was tested using the same procedure used initially. APC+ cells are in early/late stage apoptosis while PI+ cells have undergone (n=1)
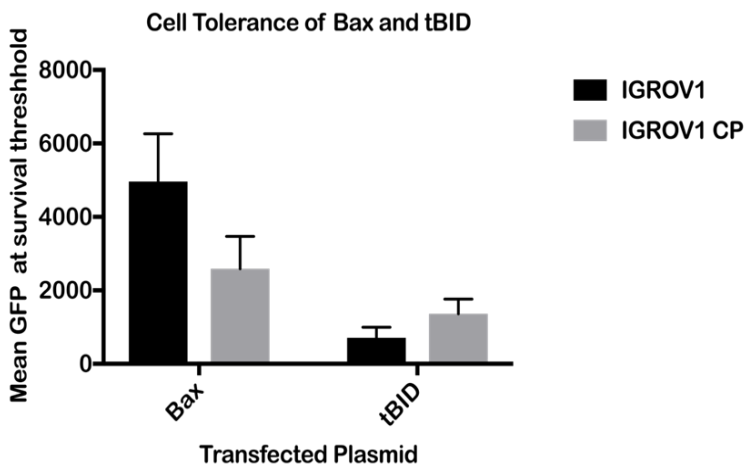
Figure 8. tBID tolerance levels induced by TRAIL treatment in IGROV1 and IGROV1 CP cell lines. GFP tagged tBID and Bax expression is shown. Bax was included as a control. The GFP levels represent the maximum quantity of tBID/Bax that 98% (survival threshold) of living IGROV1/IGROV1 CP cells can tolerate before undergoing apoptosis. A larger mean GFP value indicates that the given cell line can tolerate more of a selected protein. (n=2, error bars represent standard error of the mean).

Discussion

For over twenty years, researchers have been investigating the potential use of TRAIL as an anti-cancer agent.[7] Ovarian cancer is especially difficult to treat since drug resistance is so common.[4,17] Based on the results from our TRAIL sensitivity assay, TRAIL is a potential candidate treatment for several types of platinum-resistant ovarian cancer (Figure 2). Platinum-resistant ovarian cancer cell lines appear to be moderately sensitive to TRAIL. The fact that the majority of ovarian cancers become platinum-resistant after some time suggests TRAIL may be a successful therapy after recurrence. Studies suggest TRAIL synergizes with platinum based drugs when used in combination with TRAIL to treat ovarian cancer.[16,18] Unfortunately, TRAIL sensitivity in platinum-resistant ovarian cancer cell lines may not be very common as studies suggest TRAIL resistance is relatively abundant in ovarian cancer, yet these studies included platinum-sensitive tumors as well.[19]

While the mechanism is not well understood, it is believed that high c-FLIP levels play a role in TRAIL resistance in ovarian cancer.[16,19] Unfortunately, c-FLIP levels could not be acquired at this time for the cell lines that were tested in the experiments shown here. The role of c-FLIP may be cell line specific since some studies have found no correlation between c-FLIP and TRAIL sensitivity.[20] Studies have found a correlation between increased Akt expression and increased TRAIL resistance since Akt transcriptionally downregulates BID.[19] Results from the knockdown of BID suggest BID is important for TRAIL-induced cell death (Figure 7).

While TRAIL surface receptor expression has been suggested to influence TRAIL sensitivity,[19] these results (Figure 3) suggest that it may not be the case for IGROV1 CP and A2780 CP200 cell lines. The moderate increase in TRAIL receptors seen in PEO4 and ABTR2 cell lines requires further analysis to determine if this observation plays a role in TRAIL sensitivity (Figure 3). While only surface TRAIL receptors were quantified in the experiments above, research within the past 5 years has identified TRAIL receptors located in intracellular vesicles and the nucleus.[21] The function of these intracellular receptors is not well understood, but further study may provide mechanisms in which they influence sensitivity to TRAIL.

Investigating the native protein levels of TRAIL pathway proteins found several trends within platinum-resistant cell lines (IGROV1 CP, A2780 CP200) (Figure 4). Both BID and XIAP were upregulated in these two cell lines. Many studies performed in ovarian cancer cells suggest that XIAP does not influence TRAIL sensitivity.[19] The trend seen with FADD has not been highlighted elsewhere, so further experiments were carried out to investigate the potential role it may play in TRAIL sensitivity. An increase in caspase-8 levels as seen in IGROV1 CP cells suggests different cell lines may rely more on different proteins to initiate TRAIL-induced apoptosis (Figure 4). Further experimentation should be performed to investigate the role native caspase-8 levels may play in TRAIL signaling. The differential expression of BID seen between the two isogenic cell line pairs is an interesting result since research has suggested that tBID formation regulates TRAIL-induced apoptosis in type II cells.[13] Lower BID levels in a TRAIL sensitive cell line such as IGROV1 CP suggests that the cells may be hypersensitive to the predictably less amount of tBID that is produced via TRAIL signaling (Figure 4). However, IGROV1 CP cells were not

found to be hypersensitive and were perhaps even more resilient to tBID (Figure 8). The protein expression results highlighted the need to further investigate the roles FADD and BID play in TRAIL sensitivity.

Since FADD is located high upstream in the TRAIL pathway it was suspected that the significant difference in protein level observed would be responsible for the differences seem in TRAIL sensitivity. Native FADD mRNA levels were quantified in order to identify factors influencing the increased FADD protein levels (Figure 5). Interestingly, FADD knockdown failed to have a significant effect on TRAIL sensitivity (Figure 6). It may be the case that c-FLIP levels in the TRAIL sensitive cell lines are so low that even a small amount of FADD is sufficient for inducing apoptosis. FADD knockout experiments should be performed on the TRAIL sensitive cell lines to confirm that TRAIL-induced apoptosis is implemented through the canonical pathway, which requires FADD.

Conclusions

Several platinum-resistant ovarian cancer cells lines (IGROV1 CP, A2780 CP200, PEO4, ABTR2) are sensitive to TRAIL treatment. Understanding the mechanisms behind TRAIL sensitivity would be beneficial to treat ovarian cancer. Improved modified TRAIL delivery systems could be utilized in clinical trials containing platinum-resistant ovarian cancer patients. In the instances of TRAIL sensitivity seen above, receptor quantity does not seem to influence TRAIL sensitivity. More research into the intracellular localization of TRAIL death receptors may be beneficial. Increased FADD protein levels did not influence TRAIL sensitivity as it might have been predicted. BID knockdown caused a moderate decrease in TRAIL sensitivity indicative of type II cells. While the differential trend seen in BID levels suggested that IGROV1 CP cells may be hypersensitive to tBID, the results suggest that this is not the case. Further areas of study regarding the TRAIL pathway include the impact of post-translational modifications on caspase-8, caspase-8 subcellular localization, and the role TRAIL signaling plays in alternate pathways such as the NF-kB, JNK, and MAPK pathways.[14]

Overall, after having traveled down the TRAIL pathway and having analyzed the major proteins, the source of TRAIL sensitivity remains elusive. Future experiments should focus on identifying tumors that may be sensitive to TRAIL in order to improve clinical trials.

Materials and Methods (General)

Quantification of TRAIL sensitivity using flow cytometry. Cells were harvested after 24-hour treatment with recombinant human TRAIL. The harvested cells were exposed to PI (1:100 dilution) for 30 minutes in the dark. Annexin V was added immediately thereafter, and the samples were run using the automatic programing of the flow cytometer. Large sample sets were covered and placed on ice for longer runs.

TRAIL surface receptor quantification. Cells were harvested and centrifuged to form a pellet. The cells were counted, and 100,000 cells were transferred into a flow tube for each sample and receptor. Approximately 100 ul of 2% FBS was added to each tube. The desired antibody (DR4, DR5, FasR) was added (1:1000) to the selected tube and incubated on ice for 3 hours. After 3 hours, the antibodies were rinsed off twice using the 2% FBS and pelleted. After rinsing, the pellet was suspended in 200 ul of 4% formaldehyde. The samples were then run in the flow cytometer using the GFP metrics. Samples were gated using control cells.

Protein quantification of TRAIL pathway members. The harvested cells were lysed and dialyzed using 4M urea and 0.1% SDS (4 cycles each). Dialyzed solutions were lyophilized and the protein was diluted to 5 ug/mL. Samples were run on a polyacrylamide cell and transferred to a nitrocellulose membrane. The membranes were exposed to the desired primary antibody for at least 12 hours. The membrane was washed with PBST (2x15 minutes) then the appropriate secondary was added for at least 1 hour. The membrane was rinsed again with PBST (2x7 minutes). Film was exposed for each membrane resulting in the band pattern observed.

RNA quantification of native FADD, siFADD, and siBID mRNA levels. A Qiagen RNeasy extraction kit was used to isolate RNA for qPCR from the desired cell lines. Primers for the mRNA of interest were purchased and used in the quantity recommended by the kit protocol. Values were normalized to the value of the platinum-sensitive cell line of the isogenic pairs.

Knockdown assays using siRNA. Several different siRNAs targeting different regions of the target mRNA were purchased. The siRNA were transfected into the target cell line using electroporation. After 48-hours, the protein levels were deemed to be the lowest.

tBID/Bax hypersensitivity assay. IGROV1 and IGROV1 CP cells were harvested and centrifuged. Plasmids containing either tBID/Bax were transfected into the cells via electroporation. Approximately 48-hours after transfection, the cells were harvested and diluted to approximately 300,000 cells in a flow tube. Annexin V was added to each tube, and the samples were run alongside their negative controls. The 98th percentile of the living cell population was identified and the GFP value was recorded at that point.

Cell culture conditions. IGROV1, IGROV1 CP, A2780, and A2780 CP200 cell lines were cultured in RPMI with 10% FBS. PEO1, PEO4, and ABTR2 cell lines were cultured in DMEM with 10% FBS also containing insulin, penicillin-streptomycin-glutamine, and non-essential amino acids.

Generation of platinum/PARP inhibitor resistant isogenic cell lines. IGROV1 CP, A2780 CP200, and PEO4 cells were generated directly from their sensitive cell line.

Low dosage treatment was carried out for several months until drug testing confirmed that the cell line was resistant. The ABTR2 cell line was generated by treating PEO1 cells with low doses of Veliparib for several months. Cross-resistance to platinum-based drugs was observed along with PARP inhibitor resistance.

Acknowledgements

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