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Review Article

Targeted ovarian cancer treatment: the TRAILs of resistance

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Abstract: Ovarian cancer (OC) is the leading cause of death from gynecological malignancies. Although most patients respond to the initial therapy when presenting with advanced disease, only 10-15% maintain a complete response following first-line therapy. Recurrence defines incurable disease in most cases. Despite improvements with conventional chemotherapy combinations, the overall cure rate remained mostly stable over the years. Increased long-term survival in OC patients will only be achieved through a comprehensive understanding of the basic mechanisms of tumor cell resistance. Such knowledge will translate into the development of new targeted strategies. In addition, because OC is considered to be a heterogeneous group of diseases with distinct gene expression profiles, it is likely that different approaches to treatment for distinct sub-types will be required to optimize response. One of the new promising anti-cancer therapies is the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). TRAIL has the ability to selectively induce apoptosis in tumor cells with little toxicity to normal cells. Death receptor ligands such as TRAIL rely on the activation of the apoptotic signaling pathway to destroy tumor cells. TRAIL induces the formation of a pro-apoptotic death-inducing signaling complex (DISC) via its death receptors, TRAIL receptor 1 (TRAIL R1) and TRAIL receptor 2 (TRAIL R2). The formation of the DISC activates caspase-8 which requires further signal amplification through the mitochondrial pathway for an efficient activation of effector caspases in OC cells. The initial enthusiasm for TRAIL has been hampered by accumulating data demonstrating TRAIL resistance in various tumor types including OC cells. There is, therefore, a need to identify markers of TRAIL resistance, which could represent new hits for targeted therapy that will enhance TRAIL efficacy. In addition, the identification of patients that are more likely to respond to TRAIL therapy would be highly desirable. In this review, we discuss the different molecular and cellular mechanisms leading to TRAIL resistance in OC. In particular, we address the mechanisms involved in intrinsic, acquired and environment-mediated TRAIL resistance, and their potential implication in the clinical outcome.

Keywords: Ovarian cancer, death receptors, resistance, TRAIL

Introduction

Ovarian cancer (OC) is the fifth cause of cancer-related death in women in North America, the second most common gynecological cancer, and the leading cause of death from gynecological malignancies [1]. Although OC may arise from all cell types composing the ovaries, epithelial carcinomas arising from the surface epithelium are by far the most common (85-90% of all OC) [1]. Early detection of cancer patients remains an important objective in the field because over 70% of patients with OC are diagnosed at late stage disease, with dissemination of tumor implants throughout the peritoneal cavity [1-3]. Only 10-15% of these patients maintain a complete response to the initial therapy. The five-year survival of patients that present with late stage disease, which is the case for most patients, remains at < 30% with a mean survival of 39 months [4]. Recurrence is associated with incurable diseases in most cases. Thus, the main obstacle to an effective treatment is the failure of the initial chemotherapy to eradicate a sufficient number of tumor cells to prevent disease recurrence. In this context, deficiency in the apoptotic cascade among tumor cells is a key hallmark of OC.

The current standard treatment for advanced OC consists of cytoreductive surgery and chemotherapy. Paclitaxel combined with platinum-

based regimen is the standard first-line chemotherapy used for all patients with OC [5]. OC can be considered a chemosensitive neoplasm as the majority (80%) of patients initially respond to the combination of paclitaxel and platinum-based drugs [6]. However, 90% of the patients that initially responded will eventually develop chemotherapy-resistant diseases [6]. Although rarely curative, patients that do not respond to the first-line chemotherapy are given second-line and third-line regimens of chemotherapy in an attempt to prolong life and palliate symptoms. Despite evidence of considerable heterogeneity in their histological phenotypes and molecular profiling [7-9], most cases of OC are treated in a similar fashion. It became apparent with recent progress that the focus should be towards the development of new targeted therapies capable of exploiting the molecular and genetic characteristics of individual tumor subtypes. Clearly, the development of more effective combined initial strategies that would reduce the incidence of recurrence is highly desirable. In addition, the discovery of novel and effective therapy against chemotherapy-resistant OC is a high priority.

At the moment, there is a rapid development of novel compounds that target key components in signal transduction pathways associated with cell growth, tumor vascularity, apoptosis and invasive potential of cancer cells. One such molecule is TRAIL. The TRAIL pathway has been extensively studied in vitro and in vivo, and molecules targeting this pathway have become attractive candidates for anticancer treatment [10]. Preclinical studies in mice provided the first evidence that the soluble form of recombinant TRAIL suppresses the growth of human tumor xenografts with no apparent systemic toxicity [11]. More recently, recombinant TRAIL has entered clinical trials for the treatment of various malignancies [12, 13]. Although published phase 1/2 studies have indicated tolerated toxicity, the therapeutic efficiency was limited [14]. In addition to the soluble ligand, several agonistic antibodies targeting TRAIL R1 or TRAIL R2 have been developed and entered into clinical trials in parallel including OC patients [10, 15-18]. These agonistic antibodies may be more effective than the soluble TRAIL at eradicating tumors for several reasons, one of them being the prolonged half-life time in vivo compared to the recombinant proteins [19-22]. While soluble TRAIL can bind to all five receptors, including decoy receptors which can contribute to TRAIL resistance, selective agonistic antibodies help to avoid this unwanted effect. Another potential advantage of TRAIL and its agonistic antibodies is the fact that they induce apoptosis independently of the p53 tumor suppressor gene [10], which is frequently inactivated in OC cells. Thus, therapies targeting the TRAIL cascade may be effective against cancer cells that have acquired resistance to conventional chemotherapy via p53 inactivation. However, tumor cells have developed various mechanisms to escape the apoptosis induced by TRAIL. This underscores the need to understand the mechanisms of TRAIL resistance, and based on this knowledge, identify and validate novel combinations that could be used with TRAIL to potentiate its therapeutic efficacy. The issue of developing new combination therapies for OC is important because OC cells are prone to develop multiple strategies to evade apoptosis.

This review focuses on the TRAIL signaling cascade and the various mechanisms that OC cells may develop to suppress TRAIL cytotoxicity. The role of the cancer-associated microenvironment in TRAIL resistance is also discussed.

TRAIL and death receptors

TRAIL is a member of the TNF ligand superfamily of cytokines and it is a type II transmembrane protein expressed by cells of the immune system. The extracellular domain of TRAIL can be shed from the cell surface by cysteine proteases to produce soluble TRAIL. Both the soluble and the membrane-bounded TRAIL can trigger apoptosis by interacting with TRAIL receptors expressed by target cells. TRAIL binds to multiple receptors including TRAIL R1 (DR4), TRAIL R2 (DR5), TRAIL R3 (DcR1), TRAIL R4 (DcR2) and osteoprotegerin (OPG) [23-27]. Only TRAIL R1 and TRAIL R2 are able to transmit an apoptotic signal. Two additional receptors TRAIL R3 and TRAIL R4 act as decoy receptors and are incapable of transmitting an apoptotic signal. Both TRAIL R1 and TRAIL R2 contain death domains (DD) in their intracellular portion [28]. In contrast, TRAIL R3 lacks the DD whereas TRAIL R4 has a truncated non-functional DD and therefore both are unable to transmit an apoptotic signal. Soluble TRAIL also binds with low affinity to soluble OPG, which is a decoy receptor for RANKL that blocks the RANK-RANKL in-

teraction and limits osteoclastogenesis [29]. OPG binding to TRAIL may regulate differentiation and survival of mature osteoclast [30]. Interestingly, conditioned medium from prostate and breast cancer cells that contained high levels of OPG were found to inhibit TRAIL-induced apoptosis in vitro [31, 32]. OC ascites also contain high levels of OPG (Lane et al., unpublished data). These data suggest that OPG might potentially counteract the anti-tumor activity of TRAIL in vivo.

The selectivity of TRAIL for tumor cells can be explained, at least in part, by higher levels of TRAIL R1 and TRAIL R2 in tumor cells and the relative high levels of decoy receptors in normal cells [33, 34]. Indeed, normal cells such as human foreskin fibroblast HS27 and lung fibroblast WI38 were found to have extremely low expression of TRAIL R1 mRNA and protein, and were resistant to TRAIL-induced apoptosis [35]. In contrast to the initial belief, at least in vitro, the cytotoxic effects of TRAIL are not limited to transformed cells. For example, human normal urothelial cells [36], human prostate cells [37], human primary keratinocytes [38], and human epithelial ovarian cells [39] were shown to be susceptible or partially susceptible to TRAIL-induced apoptosis in vitro. In some of these normal cells, sensitivity to TRAIL was associated with low expression of anti-apoptotic decoy receptors [37]. Although these data raise concerns about TRAIL safety when deliver systemically; in preclinical models, recombinant soluble TRAIL has demonstrated good anticancer activity and no systemic toxicity [40]. Published phase 1/2 studies with TRAIL agonists have also generally indicated a good tolerance.

Apoptotic TRAIL signaling

There are two main pathways that initiate the apoptotic cascade: the extrinsic and the intrinsic pathway (**Figure 1**). The extrinsic pathway is triggered when TRAIL binds to TRAIL R1 or TRAIL R2. Receptor trimerization, along with the subsequent oligomerization and clustering of the receptors, leads to the recruitment of the adaptor protein Fas-associated protein with death domain (FADD). FADD allows the recruitment of the inactive pro-caspase-8 or –caspase-10 via a shared death effector domain (DED) leading to the formation of the DISC. In some cells, upon autoactivation at the DISC, activated caspase-8 and 10 cleave and directly activate the effector caspases (caspase-3, -6, -7) leading to the execution of apoptosis including membrane blebbing, inter-nucleosomal DNA fragmentation and nuclear shrinkage (type I cells) [41]. A protein called cellular FLICE-inhibitory protein (c-FLIP) shares structural homology with pro-caspase-8 and possesses a death effector domain that lacks protease activity. In specific conditions, its structure allows c-FLIP to be recruited to the DISC where it inhibits the processing and activation of pro-caspase-8. Although many isoforms of c-FLIP have been identified, only three are expressed in human cells [42]. They consist of two short variants, c-FLIP$_S$ and c-FLIP$_R$, and a long splice variant, c-FLIP$_L$. Both c-FLIP$_L$ and c-FLIP$_S$ contain two DEDs and compete with pro-caspase-8 for association with FADD [43]. Depending on the level of c-FLIP$_L$ expression, its function at the DISC will vary. When present in high amounts, c-FLIP$_L$ will exert an anti-apoptotic effect at the DISC [44]. When present in low amounts, it may heterodimerize with caspase-8 at the DISC and promotes apoptosis [45]. c-FLIP is thus seen as a major inhibitor of the extrinsic pathway of apoptosis. In other cells (so called type II cells), amplification of the signal via the intrinsic or mitochondrial pathway is necessary for efficient apoptosis. The intrinsic pathway is usually triggered in response to DNA damage, hypoxia or oncogene overexpression. As a sensor of cellular stress, p53 is a critical initiator of the intrinsic pathway. In response to cellular damage, p53 translocates from the cytoplasm to the nucleus where it promotes the transcription of pro-apoptotic members of the Bcl-2 family. Pro-apoptotic Bcl-2 family members such as Bax and Bak form pores in the outer mitochondrial membrane causing the release of cytochrome c and other apoptogenic factors such as apoptosis inducing factor (AIF) and SMAC/DIABLO into the cytoplasm. Cytochrome c, along with apoptosis protease activating factor-1 (APAF-1) and pro-caspase-9 form the apoptosome. Within the apoptosome, clustered pro-caspase-9 gets activated and it cleaves downstream effector caspases, leading to the hallmarks of apoptosis [46]. The release of SMAC/DIABLO from the mitochondria promotes apoptosis by binding to and neutralizing members of the family of inhibitor of apoptosis proteins (IAPs), which can block caspase-3 activity through its baculovirus IAP repeat domains. Although the extrinsic and intrinsic pathways are activated by different mechanisms, these two pathways are interconnected (**Figure**

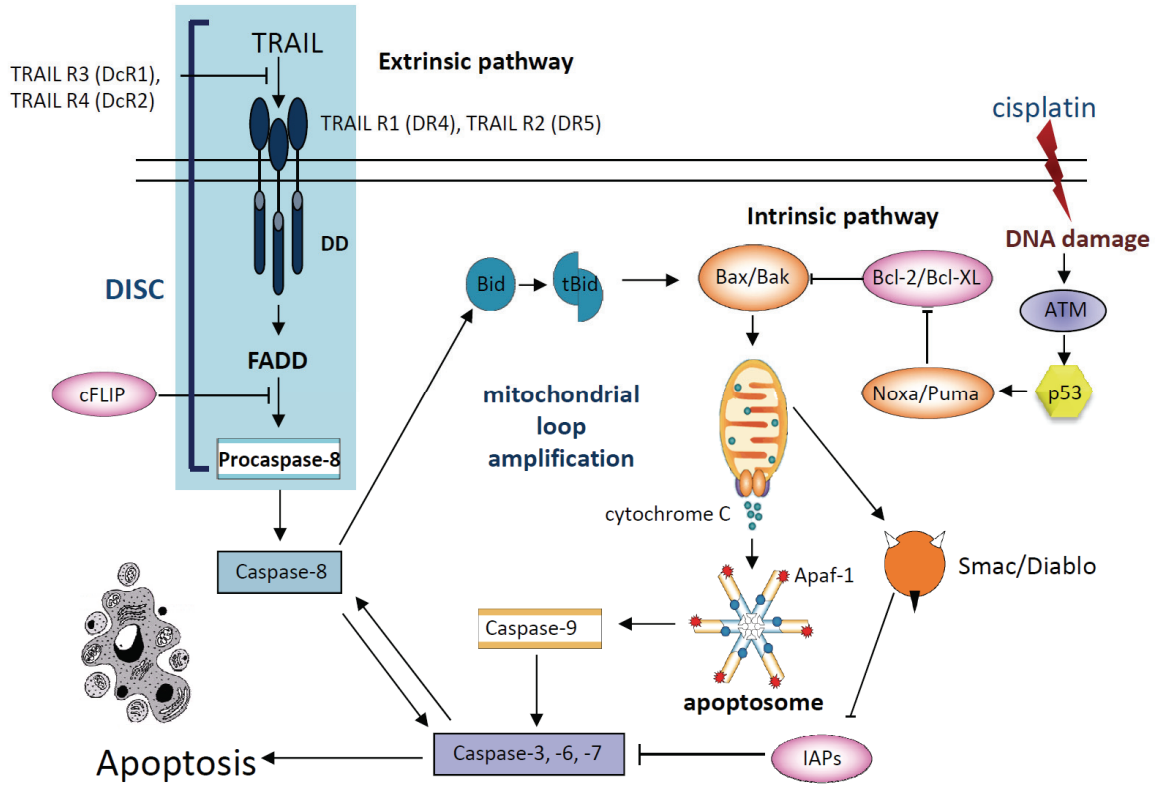

Figure 1. Apoptotic TRAIL signaling. Binding of TRAIL to death receptors (TRAIL R1, TRAIL R2) leads to the recruitment of the adaptor molecule, FADD. Pro-caspase-8 binds to FADD leading to DISC formation and resulting in its activation. Activated caspase-8 directly activates executioner caspases (caspase-3, -6, and -7) (type I cells) or cleaves Bid (type II cells). Translocation of the truncated Bid (tBid) to the mitochondria promotes the assembly of Bax-Bak oligomers and mitochondria outer membrane permeability changes. Cytochrome c is released into cytosol resulting in apoptosome assembly. Active caspase-9 then propagates a proteolytic cascade of effector caspases activation that leads to morphological hallmarks of apoptosis. Further cleavage of pro-caspase-8 by effector caspases generates a mitochondrial amplification loop that further enhances apoptosis. When FLIP levels are elevated in cells, caspase-8 preferentially recruits FLIP to form a caspase-8-FLIP heterodimer which does not trigger apoptosis.

1). In type II cells, activated caspase-8 cleaves pro-apoptotic Bcl-2 family member Bid to form truncated Bid (tBid), which can then interact with Bax/Bak. This interaction increased the release of cytochrome c from the mitochondria. Thus, Bid provides a connection between extrinsic and intrinsic pathways (so called mitochondrial amplification step). The reasons that determine whether tumor cells rely of type I or II signaling is not well understood. It has been proposed that the levels of c-FLIP and XIAP relative to caspase-8 and SMAC/DIABLO might be important determinants [35].

Bcl-2 family proteins are involved in the regulation of apoptosis by controlling mitochondrial membrane permeability. Several studies have demonstrated that these proteins can interact with each other and these interactions can neutralize their pro- or anti-apoptotic functions. The balance between anti- and pro-apoptotic members dictates the fate of cell survival or death. Pro-apoptotic Bcl-2 members can be divided into 2 groups according to their function and the number of BH domains that they possess. Proteins containing BH domains 1-3 are known as multidomain pro-apoptotic proteins such as Bax, Bak and Bok [47]. BH-3-only pro-apoptotic proteins such as Bik, Bid, Bad, Bim, Bmf, Noxa, Puma and others can form homo- and/or hetero -dimers with the multidomain proteins Bax and Bak to promote their activation. Anti-apoptotic proteins such as Bcl-2, Bcl-XL and Mcl-1 can also form hetero-dimeric interactions with Bax

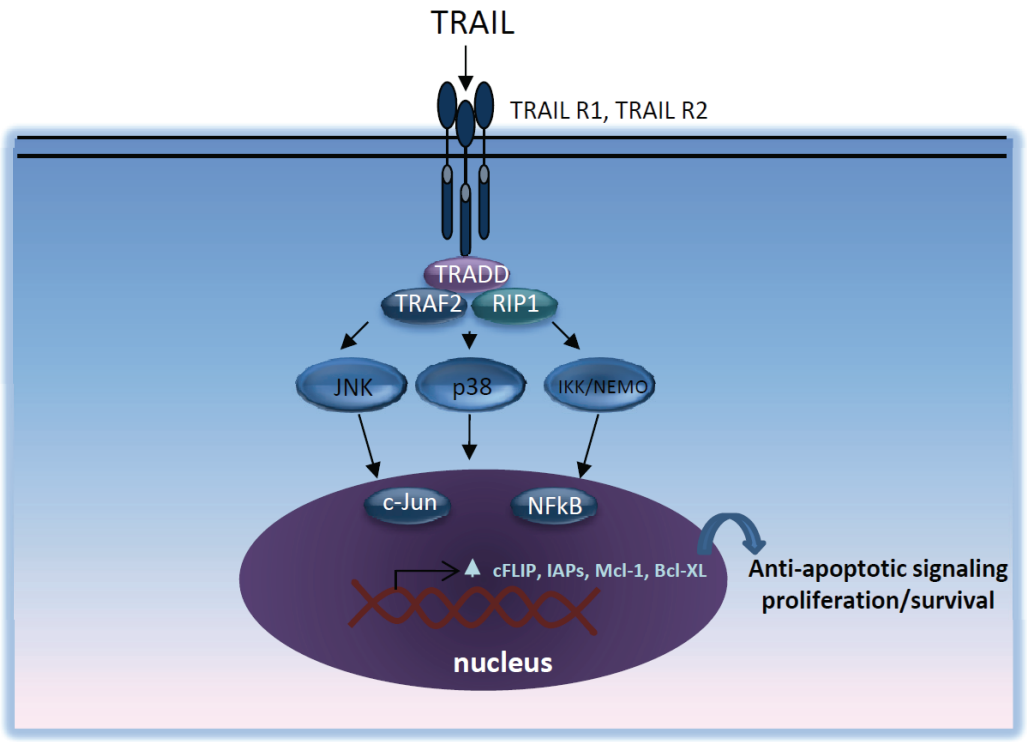

Figure 2. Non-apoptotic TRAIL signaling. In cells that resistant to TRAIL-induced apoptosis, TRADD, TRAF2 and RIP1 bind to the receptor. This complex signals through NF-κB, p38 and JNK pathway resulting in the up-regulation of target genes involved in anti-apoptotic function and proliferation.

and Bak, thereby neutralizing their pro-apoptotic activity. Anti-apoptotic proteins can form heterodimers with BH-3-only proteins and this interaction may neutralize the pro-survival function of anti-apoptotic proteins. Thus, TRAIL-induced apoptosis can be regulated at multiple levels involving both the extrinsic and intrinsic pathways (**Figure 1**).

A number of studies showed that TRAIL can also trigger non-apoptotic pathways [48]. Under certain circumstances, engagement of TRAIL receptors can lead to the formation of a secondary signaling complex by promoting the recruitment of RIP1, TRAF2 and NEMO/IKK-γ, which is capable of activating nuclear factor-κB (NF-κB) and MAP kinase pathways thereby promoting cell survival [49] (**Figure 2**). NF-κB activation promotes the transcription of anti-apoptotic proteins such as Mcl-1, IAPs and c-FLIP leading to the inhibition of apoptosis. Recently, it was demonstrated that myc oncogene decreases the expression of Mcl-1 and IAPs by blocking TRAIL-mediated NF-κB activation [50]. Myc can also transactivate TRAIL R2 or suppress the transcription of c-FLIP thereby enhancing TRAIL-induced apoptosis [51]. The biological implications of alternative TRAIL signaling cascades remain controversial and further work is required to understand how this could influence the outcome of ongoing trials with TRAIL or its agonists.

TRAIL cytotoxicity in ovarian cancer

The therapeutic potential of TRAIL has been evaluated in OC. Early studies have shown that OC cell lines displayed variable sensitivity to recombinant TRAIL [39, 52-54]. Although TRAIL resistance was a common finding in these studies, interestingly, resistance to chemotherapy did not correlate with TRAIL resistance. This suggests that TRAIL could be useful in patients that have developed resistant disease to cisplatin and paclitaxel [55]. Furthermore, several studies demonstrated that the combination of TRAIL with cisplatin was more efficient than either molecule alone [52-56]. One possible

explanation for these findings is the fact that cisplatin was shown to up-regulate cell surface expression of TRAIL receptors in tumor cells [54]. Conflicting results were reported regarding the prognostic value of TRAIL expression in OC cells. Lancaster et al. reported that high TRAIL expression in tumor cells was associated with a more favorable outcome in a cohort of 120 patients [57]. In contrast, Horak et al. did not find such an association in a smaller cohort of 68 patients [58]. However, they showed using tissues microarray immunostaining that stromal TRAIL expression was a strong predictor of overall survival [58]. Interestingly in this study, almost 50% of the analyzed tumors expressed elevated levels of c-FLIP$_L$ and about 80% of tumors displayed low expression of TRAIL R1 and/or TRAIL R2, which could contribute to the protection of OC cells from TRAIL-induced apoptosis. Furthermore, this group reported that epigenetic silencing of TRAIL R1 occurred in 8% to 27% of OC tumor samples [59]. In another study, higher expression of TRAIL R1 in OC cells isolated from ascites was associated with a worse outcome [60]. Although these data may suggest that TRAIL sensitivity is altered in a significant proportion of ovarian tumors, recent evidence demonstrated that TRAIL agonists can induce substantial cytotoxicity in ovarian tumors. Estes et al. evaluated the cytotoxicity of TRAIL R2 monoclonal antibody (TRA-8) in nineteen chemotherapy-naive primary ovarian tumor samples (stage III/IV) [61]. They showed that TRA-8 induced dose-dependent cytotoxicity in most samples tested. Increased cytotoxicity was observed when TRA-8 was used in combination with chemotherapeutic drugs [61]. The potential of TRA-8 was further evaluated in a xenograft mouse model of OC. When used alone, TRA-8 produced only a modest benefit in terms of tumor growth inhibition [61]. However, animals treated with the combination of carboplatin, docetaxel and TRA-8 demonstrated a better outcome when compared to carboplatin and docetaxel only. Enhanced tumor cell apoptosis and survival of treated animals has also been observed when soluble TRAIL was combined with cisplatin in an OC xenograft mouse model [22].

Because TRAIL cytotoxicity in OC cells relies on the activation of both the extrinsic and the intrinsic apoptotic pathways, the combination of TRAIL with growth factor inhibitors [52, 62], molecules that modulates pro- or anti-apoptotic proteins [63-65], and proteasome inhibitors has been evaluated [66]. Many studies have shown that proteasome inhibitors are able to induce apoptosis in various tumor cells both in vitro and in vivo [67, 68]. These studies provided the basis for introducing proteasome inhibitors into the clinic, notably for multiple myelomas and mantle cell lymphomas [68, 69]. In addition, there is evidence in various tumor models that proteasome inhibitors enhance the sensitivity of tumor cells to TRAIL [70-72]. Several studies have shown that proteasome inhibitors enhance TRAIL-induced apoptosis in OC cell lines and primary OC cells [73, 74]. The combination of proteasome inhibitors and TRAIL agonists could offer a new therapeutic strategy for patients with refractory advanced OC.

The enhanced efficacy of TRAIL in combination with other agents in preclinical models is encouraging and suggests that combination therapies with TRAÌL probably represent the best clinical option at this point. As it will be discussed below, given the various pathways that may contribute to TRAIL resistance in OC, a combination of molecules that targets critical steps in the TRAIL signaling cascade is likely to be the most efficient approach.

Mechanisms of TRAIL resistance in OC cells

Intrinsic resistance

Intrinsic resistance is observed when tumor cells are resistant to a specific drug without previous exposure to this drug. These tumor cells are thus inherently resistant to TRAIL. As stated above, intrinsic TRAIL resistance among OC cell lines and primary ovarian cancer cells has been frequently observed [39, 52-54]. TRAIL resistance has been reported in approximately 50% of tested tumor cells, including OC cells. Not surprisingly, no unique mechanism underlying TRAIL resistance has been observed so far in OC cells. Susceptibility to TRAIL-induced apoptosis can be regulated at multiple levels in the apoptotic signaling cascade. It has been reported that lack of expression of TRAIL R1 due to epigenetic silencing correlated with resistance to TRAIL-induced apoptosis in OC cells [59]. In contrast, Shivapurkar et al. found no aberrant methylation in the promoter of TRAIL R1 and TRAIL R2 in a subset of 23 OC samples [75]. However, aberrant methylation of TRAIL R4 or TRAIL R5 was observed in 40% of the OC

in this study. Despite these data, the levels of TRAIL receptors or decoy receptors do not usually correlate with sensitivity or resistance to TRAIL in OC cell lines [39]. However, the modulation of TRAIL receptors expression may sensitize tumor cells to TRAIL. For example, celestrol-induced upregulation of TRAIL R1 and TRAIL R2 enhances TRAIL-induced apoptosis [76].

Intracellular molecules acting downstream of TRAIL receptors also contribute to the resistance of OC cells. As found in other cancer cells, c-FLIP expression modulates the sensitivity of OC cells to TRAIL-induced apoptosis and has been associated with intrinsic TRAIL resistance. A number of studies have demonstrated that the down-regulation of c-FLIP$_L$ (through different means) enhances TRAIL-induced apoptosis in resistant OC cells [39, 62, 77-79]. In addition, the knockdown of c-FLIP$_L$ inhibited human OC cell lines migratory phenotype in a TRAIL-dependent manner in vitro and inhibited the invasion of tumor cells into the peritoneal cavity [80]. Elevated expression of c-FLIP$_L$ has been reported in a substantial percentage of OC tissues from patients with advanced diseases [58, 81] and has been associated with adverse outcome in some studies [82, 83] whereas others have found no such association [84].

Constitutively active Akt promotes cell survival and resistance to chemotherapy. The constitutive activation of Akt in OC cell lines and primary tumor cells also promotes resistance to TRAIL [85]. There is a close correlation between the activation of Akt in OC cells and the degree of resistance to TRAIL [85, 86]. The inhibition of Akt phosphorylation reversed cellular resistance to TRAIL whereas the transfection of Akt in tumor cells with low Akt basal activity enhanced TRAIL resistance [85]. The authors identified a novel mechanism of Akt-mediated inhibition of TRAIL-induced apoptosis. They showed that Akt transcriptionally regulates the expression of Bid [85]. Cells with high Akt activation express lower Bid protein levels and are thus more resistant to TRAIL-induced apoptosis. In addition, the knockdown of Bid in sensitive OC cells significantly enhanced their resistance to TRAIL-induced apoptosis. The role of Akt in TRAIL resistance among OC cells is also supported by the observation that the inhibition of Akt activation by trastuzumab [87], an erbB-2 receptor inhibitor, or by a small molecule that inhibits hPEBP4 [88], enhanced TRAIL-induced apoptosis. This correlation between the state of Akt activation and TRAIL resistance has been reported in other types of cancer, notably prostate cancer cells [89, 90].

As stated previously, in OC cells, TRAIL triggers changes in mitochondrial membrane permeability which results in the release of pro-apoptotic proteins such as cytochrome c and SMAC/DIABLO from the mitochondria. In a cohort of 75 patients, Mao et al. demonstrated decreased expression of SMAC/DIABLO and increased expression of XIAP in OC compared to normal ovarian tissues [63]. However, they observed no difference in SMAC/DIABLO and XIAP expression between TRAIL sensitive and resistant cell lines. To assess the biological relevance of these observations, they stably transfected TRAIL resistant OC cell lines with a SMAC/DIABLO expression vector and showed enhanced TRAIL-induced apoptosis in transfected cells. Similarly, the treatment of TRAIL resistant OC cells with a small molecule SMAC/DIABLO mimic enhanced TRAIL- and TRAIL R1 or R2 agonist-induced apoptosis [64]. Others have found a lack of correlation between XIAP protein expression and TRAIL sensitivity [85]. Furthermore, down-regulation of XIAP in TRAIL resistant OC cells failed to enhance TRAIL-induced apoptosis [85] suggesting that XIAP is not a major factor contributing to TRAIL resistance in OC.

Not all OC cells are sensitive to TRAIL-induced apoptosis which might limit its therapeutic potential. Intrinsic TRAIL resistance appears to be multi-factorial and can be influence by the activation of survival pathways such as Akt. In this context, the identification of informative and validated biomarkers of TRAIL resistance will be important for selecting patients and predicting the clinical outcome.

Acquired resistance

Acquired resistance is a mechanism by which tumor cells that were initially sensitive to a drug adapted to survive to prolonged exposure to this drug. This type of resistance is believed to be caused by sequential genetic alterations in tumor cells that eventually result in a therapy-resistant phenotype. Understanding how tumor cells may acquired TRAIL resistance over the course of treatment is especially relevant now that TRAIL agonists are being evaluated in phase I/II clinical trials. Theoretically, any block

in the intrinsic or extrinsic pathway that develops during TRAIL treatment could lead to acquired resistance. Not surprisingly, most studies investigating the mechanisms of TRAIL resistance have focused on intrinsic resistance. Indeed, studies that have directly addressed the problem of acquired TRAIL resistance are limited. Nonetheless, it is important to investigate this mechanism of resistance because the molecular alterations responsible for intrinsic TRAIL resistance may be different from those of acquired resistance. The most common strategy that has been employed so far to study acquired TRAIL resistance has consisted of exposing a sensitive cancer cell line to increasing sub-lethal concentrations of TRAIL or its agonists in vitro. Using such an approach with a colon cancer cell line, Jin et al. demonstrated that the levels of cell surface TRAIL R1 were significantly down-regulated in TRAIL resistant cells despite no change in TRAIL R1 mRNA or protein expression suggesting an alteration in receptor transport to the cell membrane [91]. Interestingly, these TRAIL resistant cells were not cross-resistant to either FasL or paclitaxel. Decreased cell surface expression of TRAIL R1 and/or TRAIL R2 has also been observed in studies involving HL60 leukemia cells [92, 93] and breast cancer cells [94]. Wang et al. reported that acquired TRAIL resistance in H460 lung cancer cells was associated with enhanced expression of FLIP and Mcl-1 [95]. FLIP recruitment to the DISC inhibited the recruitment and the activation of pro-caspase-8 whereas Mcl-1 up-regulation suppressed mitochondrial apoptosis. The ratio of pro-caspase-8 to FLIP was also found to determine the level of pro-caspase-8 recruitment and activation at the DISC in hepatocellular carcinoma cells [96]. Interestingly, this study demonstrated that although TRAIL R2 was upregulated in resistant cell lines, increased TRAIL R2 expression was insufficient to induce apoptosis. In another study, acquired TRAIL resistance was the results of the combination of decreased TRAIL R1 and TRAIL R2 cell surface expression and increased FLIP and Stat5 expression [94]. Stat5 proteins are members of the STAT family of transcription factors that mediate cytokine- and growth factor-induced anti-apoptotic signals [97]. Song et al. showed that Bcl-X_L was a critical factor in the development of acquired TRAIL resistance in prostate cancer cells [98]. Acquired resistance to TRAIL may also result from alterations in multiple key apoptotic mediators that affect both the intrinsic and the extrinsic pathways. For example, Zhang et al. demonstrated that TRAIL-selected resistant melanoma tumor cells expressed markedly lower levels of TRAIL R1, TRAIL R2, pro-caspase-8 and pro-caspase-3, and decreased expression of a number of pro-apoptotic Bcl-2 family members such as Bid, Bim, Puma, Noxa and Bad as compared to the unselected parental cell line [99]. Using a pair of isogenic colorectal carcinoma cell lines that were derived from the primary tumor and from a metastatic lymph node from the same patient six months later, Ndozangue-Touriguine et al. showed that acquisition of TRAIL resistance in the metastatic cells resulted from a mitochondrial block that prevented mitochondrial outer membrane permeability (MOMP) [100]. Interestingly, although TRAIL-induced caspase-8 activation resulted in the cleavage of full-length Bid into the active truncated from of Bid (tBid) in TRAIL-resistant cells, tBid relocalization to the mitochondria failed to induce MOMP. In addition, the authors found that XIAP down-regulation led to TRAIL sensitization of resistant cells [100]. All together, these studies demonstrate that the mechanisms of acquired resistance to TRAIL can be due to the inhibition of both extrinsic and intrinsic apoptotic cascades, and are heterogeneous among different tumor types.

Little is known about the molecular mechanisms underlying acquired TRAIL resistance in human OC cells. Lane et al. demonstrated that TRAIL acquired resistance was due to a rapid degradation of active caspase-3 subunits by the proteasome in the TRAIL resistant variant OC cells OVCAR3 [101]. Not surprisingly thus, TRAIL resistant cells were also cross-resistant to FasL. These authors did not find any difference in TRAIL receptor expression between sensitive and resistant cells. TRAIL resistant OVCAR3 cells remained however sensitive to chemotherapeutic drugs. In addition, the expression of different pro- and anti-apoptotic Bcl-2 family members did not significantly change in cells that acquired TRAIL resistance. Li et al. exposed a TRAIL-sensitive OC cell line to low and repeated doses of TRA-8, a TRAIL R2 agonist antibody, and the resultant TRA-8 resistant cell line displayed a selective defect in TRAIL-R2 signaling cascade [102]. Interestingly, the apoptotic responses induced by TRAIL, a TRAIL-R1 agonist antibody (2E12), and in other apoptotic stimuli were not impaired. One reassuring finding of these studies in OC and other in different tumor

types is the fact that TRAIL resistant cells remain sensitive to standard chemotherapy. In fact, combining standard chemotherapy with TRAIL treatment appears to be beneficial because treatment with cisplatin or carboplatin upregulates the expression of TRAIL death receptors regardless of the p53 status which leads to increase apoptosis in OC cells [103].

Whether these in vitro studies on acquired TRAIL resistance are relevant to cancer patients remain to be determined. After completing clinical trials with TRAIL agonistic antibodies, it will be important to investigate whether TRAIL resistance has developed in non-responders and determine how this resistance has evolved during treatment. Acquired TRAIL resistance could be especially relevant in clinical settings because it may switch the cytotoxic beneficial effect of TRAIL into a detrimental effect by stimulating tumor cell proliferation. Acquired TRAIL resistance in tumor cells has been associated with increased migration, invasion and metastasis [104-106].

It is possible that the population of resistant cells that emerge during the selective process actually results from a subset of parental cells that were intrinsically resistant to TRAIL. In this context, increasing evidence suggests that OC stem cells are present, albeit in a very small percentage, in various cell lines such as OVCAR3, SKOV3 and IGROV1 as well as in OC ascites [107]. These progenitor stem cells are more resistant to cisplatin and paclitaxel. They could contribute to the emergence of drug resistant tumor cells during the treatment of OC.

Environment-mediated resistance

Most studies have been performed in unicellular models to characterize TRAIL resistance mechanisms. This approach however does not take into account the interactions that exist between host and tumor cells. Unlike most other solid cancers where the stroma surrounding tumor cells constitute the microenvironment, the accumulation of peritoneal fluid that develops during OC progression, which contains floating tumor cells, represents a unique form of tumor microenvironment. The floating malignant cells are capable of surviving and proliferating despite lacking immediate proximity to blood vessels presumably due to the permissive attributes of this microenvironment. The incidence of ascites in women presenting with OC ranges from 45% to 75% depending on the tumor type [108]. The presence of ascites is generally regarded as a grave prognostic sign [109]. OC cells have the ability to survive in ascites despite the absence of matrix anchorage. There are several indirect evidences to suggest that ascites influence the progression of OC. Characterization of tumor cells from ascites using immunohistochemistry (IHC) identified a series of molecules that are differentially expressed between ascites tumor cells and tissues [110]. More recently, proteomic profiling of tumor cells from ascites before and after chemotherapy showed an increase in the activation of survival pathways such as Akt pathway, suggesting that the tumor microenvironment influences the drug resistance development [111]. Stromal myofibroblasts and endothelial cells, adjacent to cancer cells in solid tumors, are replaced by mesothelial cells and by a variety of immune cells in ascites. In this microenvironment, mesothelial cells exist in an activated state and they may appear phenotypically and functionally similar to tumor cells [112]. Tumor-infiltrating lymphocytes (TIL) usually represent a major component of immune cells. Unfortunately, these cells are largely ineffective in arresting tumor growth. Although many of these TIL cells are specific for tumor-associated antigens, they show very limited cytolytic activity against autologous tumor cells [113]. Natural killer (NK) cells are present in ascites and about 60% of infiltrating NK cells in tumors expressed TRAIL at their surface [103]. In mice bearing tumors depleted of NK cells, TRAIL increased tumor growth and abolished the cytotoxic effect of TRAIL R2 agonist antibody suggesting that, at least in this model, selective immune cells possess antitumor activity.

Environment-mediated drug resistance (de novo resistance) is a form of resistance by which tumor cells are transiently protected from chemotherapy-induced apoptosis via the induction of survival signaling pathways [114]. Soluble factors in the tumor microenvironment engage cell surface receptor to activate survival pathways (**Figure 3**). The acellular fraction of ascites is a complex inflammatory fluid containing growth factors [115-117], lysophosphatidic acid (LPA) [118, 119], cytokines [113, 120, 121] and extracellular matrix (ECM) constituents [122]. Ascites fluid from patients with pancreatic or ovarian cancer contains significant levels of LPA,

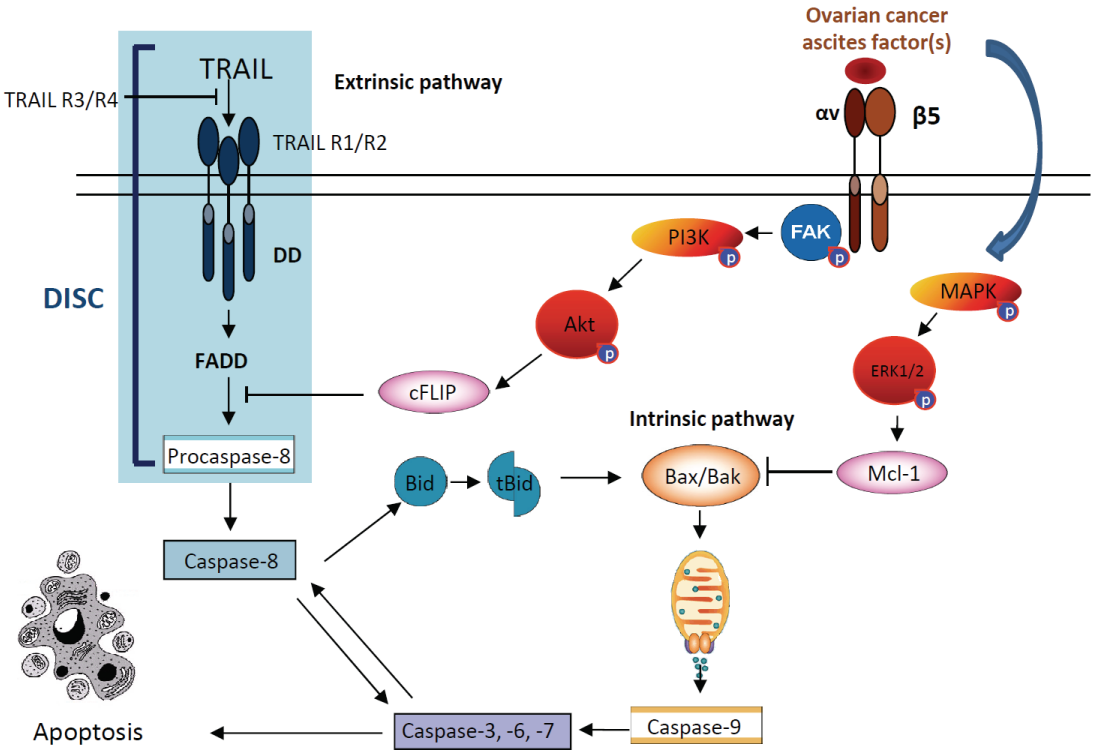

Figure 3. Model for ovarian cancer ascites-mediated protection from TRAIL-induced apoptosis. TRAIL binding to death receptors TRAIL-R1 and TRAIL-R2 results in death- inducing-signaling complex (DISC) formation, activation of procaspase-8, Bid cleavage, release of cytochrome c from mitochondria, and caspase-9 and caspase-3 activation. Binding of ascites survival factors to avβ5 integrin leads to the phosphorylation of FAK and Akt. Akt activation up-regulates the levels of c-FLIP protein leading to inhibition of caspase-8. Ascites also activate the ERK pathway leading to up-regulation of the anti-apoptotic Mcl-1 protein.

which exceed levels required to activate LPA receptors [86, 119]. LPA, one of the ligands of G -protein coupled receptors, has been shown to induce cell survival signaling pathways through PI3K/Akt in OC cells [123], and to inhibit cisplatin-induced apoptosis [124]. LPA may bind to four distinct receptors LPA1-4. Among these receptors, LPA2, LPA3 and LPA4 are aberrantly expressed in OC cells [125]. LPA receptors can couple to at least three distinct G proteins (G_q, G_i, and $G_{12/13}$). Activation of the PI3K/Akt pathway has been predominantly linked to the activation of G_i by LPA. LPA in pancreatic ascites is an important factor for the migration and invasion of pancreatic cancer cells in vitro [119, 126]. LPA protects from TRAIL-induced apoptosis by regulating the expression of DR4 or c-FLIP [124, 127]. However, in the context of ovarian ascites, the blockade of LPA cascade did not impact on TRAIL-induced apoptosis in OC cells [86]. In addition, LPA did not protect OC cells from TRAIL-induced apoptosis in vitro [86].

Although a wide variety of cytokines can be measured in OC ascites, interleukin-6 (IL-6) and interleukin-8 (IL-8) are among the most abundant [113, 120]. The concentration of these cytokines in ascites is 40- to 500-fold higher as compared to the levels found in serum [113]. IL-6 can be secreted in ascites by ovarian cancer cells, tumor-associated macrophages and peritoneal mesothelial cells. However, levels of IL-6 secreted by activated mesothelial cells are 600-fold higher than those secreted by ovarian cancer cells [128]. The source of the IL-8 found in ascites has not been well defined. These pro-inflammatory cytokines are involved in different pathophysiological processes including carcinogenesis. In OC, IL-6 is thought to be involved in host immune responses to the disease [129-131]. IL-6 has also been demonstrated to be involved in autocrine growth of OC cells [132-134]. IL-6 signaling in OC cells can regulate tumor cell proliferation, invasion and angiogenesis [135-137]. IL-8 was recently reported to pro-

mote ovarian tumor growth in vivo [138]. A number of studies have reported an association between serum levels of IL-6 and prognosis, where elevated levels correlated with a poor relapse-free and overall survival [140, 141]. However, others have not found such correlation between elevated serum levels of IL-6 and patient's survival [142]. Interestingly, it was recently shown that elevated ascites levels of IL-6, but not IL-8, were an independent predictor of shorter progression-free survival [120]. Whether IL-6 is a critical soluble factor in ascites-mediated TRAIL resistance is unclear. However, recent data showing that recombinant IL-6 does not protect from TRAIL-induced apoptosis in OC cells in vitro and that IL-6 depletion from OC ascites does not affect their prosurvival properties suggest that IL-6 is not a critical factor in ascites-mediated TRAIL resistance (Lane, unpublished data).

In addition to cytokines and growth factors, tumor host interactions include tumor cells-ECM communications. ECM components bind to cell surface integrins; a family of heterodimers consisting of non-covalently linked α and β subunits [142]. Different integrins exhibit different ligand specificities. Integrins transmit signals directly through ligation-dependent recruitment of non-receptor tyrosine kinases from the focal adhesion kinase (FAK) leading to the activation of several cell signaling pathways including the PI3K/Akt pathway [143]. The role of integrins in mediating cell proliferation, migration and survival in ovarian cancer is well established [86, 144-146]. Based on the variety of soluble factors that composed the ascites, one can therefore expect an important interplay between OC cells and the microenvironment that may promote tumor cell survival and tumorigenesis. It is also expected that the microenvironment undergoes changes in response to emerging tumor cells which can further promote cancer progression.

Alternatively, homotypic or heterotypic tumor cells adhesion or adhesion to ECM components mediates the activation of survival pathways. By definition, de novo resistance persist as long as tumor cells are in contact with the microenvironment. Once the microenvironment is removed, tumor cells usually revert to a sensitive phenotype. As stated above, the ovarian cancer ascites contains a number of candidate soluble factors that could promote the emergence of TRAIL resistance. Recently, it has been shown that the PI3K/Akt cascade is activated by ovarian cancer ascites which contributes to TRAIL resistance [86, 147]. The ability of different ascites to induce Akt phosphorylation in tumor cells strongly correlates with their ability to inhibit TRAIL-induced apoptosis [86]. The PI3K/Akt pathway most likely couples signals from ascites-activated cell surface receptors which regulate the expression and/or phosphorylation of apoptosis-regulating targets. Indeed, after ascites-induced activation of αvβ5 integrins, the focal adhesion kinase (FAK) is phosphorylated and FAK induces the activation of Akt [86]. This leads to Akt-mediated up-regulation of c-FLIPs expression in ovarian cancer cells (**Figure 3**) [86, 148]. The Raf/MEK/ERK pathway is another cascade that plays critical roles in the transmission of signals from cell surface receptors to regulate gene expression. For example, OC cells incubated with ascites displayed rapid ERK phosphorylation which results in Mcl-1 up-regulation (Goncharenko-Khaider and Lane, unpublished data).

Collectively, these data demonstrate the importance of ascites as a microenvironment that supports tumor cell growth and resistance to therapy. Directly targeting ascites-induced survival pathways in tumor cells or blocking soluble factor-mediated activation of these pathways might be an effective approach to enhance TRAIL cytotoxicity.

Conclusions and future directions

The inherent properties of TRAIL or its agonists offer a new targeted therapy for OC. Preclinical studies using TRAIL or its agonists have demonstrated the therapeutic potential of these molecules and formed the basis of ongoing phase I/II clinical trials. Although these treatments appear to be clinically well tolerated so far, intrinsic, acquired and environment-mediated resistance may limit the effectiveness of these approaches. However, the development of combination treatments appears to be capable of overcoming, at least in part, some of these limitations. Increasing understanding of the "TRAILs" of resistance in OC is opening the path for therapeutic approaches that exploit critical regulators of resistance for the development of new combined therapies. As the search for more effective treatment for OC continues, the morbidity and mortality will hopefully improve.

Significant progress has been made in our understanding of the molecular basis of TRAIL resistance in OC and efforts should continue to further improve this knowledge as this will likely lead to the development of specific biomarkers of resistance and more efficient targeted therapies.

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