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Review

Chemoresistance in human ovarian cancer: the role of apoptotic regulators

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Abstract

Ovarian cancer is among the most lethal of all malignancies in women. While chemotherapy is the preferred treatment modality, chemoresistance severely limits treatment success. Recent evidence suggests that deregulation of key pro- and anti-apoptotic pathways is a key factor in the onset and maintenance of chemoresistance. Furthermore, the discovery of novel interactions between these pathways suggests that chemoresistance may be multi-factorial. Ultimately, the decision of the cancer cell to live or die in response to a chemotherapeutic agent is a consequence of the overall apoptotic capacity of that cell. In this review, we discuss the biochemical pathways believed to promote cell survival and how they modulate chemosensitivity. We then conclude with some new research directions by which the fundamental mechanisms of chemoresistance can be elucidated.

Ovarian Cancer and Chemoresistance: A Molecular and Cellular Perspective

Although ovarian cancer ranks seventh among all cancers in women in terms of prevalence, almost 60% of those who have ovarian cancer eventually succumb to the disease. In 2002, 23,300 new cases were identified and 13,900 deaths were reported in the United States alone. While breast cancer has a much higher incidence (203,500 new cases), it has a considerably lower mortality rate (39,600 deaths; approximately 20% mortality rate) when compared to that of ovarian cancer [1]. In fact, ovarian cancer causes more deaths than any other cancer of the female reproductive system. Much of the failure to obtain better cure rates in ovarian cancer is a consequence of late diagnosis; only about 25% of patients are diagnosed when the disease is still confined to the ovary. At advanced stages (stages III and IV), when the disease has spread beyond the ovary, treatment becomes increasingly ineffective.

Currently, the preferred treatment regimen for ovarian cancer is combination chemotherapy; usually a platinum-based drug, such as cisplatin or carboplatin, coupled with paclitaxel. While this treatment course shows promising effects in a high percentage of cases, the development of chemoresistance is a hurdle that significantly hinders successful treatment outcomes [2]. The ability of a cancer cell to respond to a chemotherapeutic agent is believed to be due, in part, to its apoptotic capacity. Moreover, it is accepted that the process of drug-induced apoptosis is governed not only by the upregulation of pro-apoptotic factors or tumor suppressors, but also by modulation of

cell survival factors. To that end, a number of genes involved in either the induction or inhibition of apoptosis, namely the p53, Akt, and phosphoinositol-3-OH-kinase (PI3K) gene families, are aberrantly regulated in ovarian cancer [3,4]. Because of their wide-ranging biological effects, deregulation of one or more of these factors may give rise to a failure of drug-induced apoptosis.

As such, a number of groups have focused their research on elucidating the precise mechanisms by which chemotherapeutic agents induce, and by extension fail to induce, apoptosis in ovarian cancer cells. We and others have been particularly focused on elucidating the mechanisms by which several key apoptotic regulators, including p53, the Inhibitor of Apoptosis (IAP) family, the Akt family, and the death-receptor family, may influence the response of ovarian cancer cells to cisplatin. We have found that aberrant regulation of these pathways plays a significant role in the induction and maintenance of chemoresistance. This review first outlines some of the major regulatory pathways implicated in control of cell fate. We then elucidate some of the recently established interactions between these apoptotic regulators, and show how these interactions are critical for chemoresistance. Finally, we explore some potential avenues for future work in this area.

The effectiveness of many of the chemotherapeutic agents used in human cancer is highly dependent upon the ability of the cancer cell to undergo drug-induced apoptosis. It is now widely held that the decision to live or die in response to a given agent is a reflection of the overall apoptotic balance within a given cell. For example, cisplatin has been shown to upregulate the pro-apoptotic factors p53, Fas, and Bax in a number of cell types [5–7]. However, it also down-regulates specific cell survival factors such as Xiap and Akt [8]. Recent evidence suggests that chemoresistance may represent an overall imbalance between these two phenomena. To that end, we and others have long been interested in elucidating the mechanisms of action of cisplatin in terms of its ability to influence these cell survival and death pathways. These studies have identified a number of signaling pathways that are differentially regulated between chemosensitive and chemoresistant ovarian cancer cells. We will outline some of these pathways, and their potential cross-talk hereafter.

Cell survival factors

While apoptosis is an important mechanism in normal development and prevention of abnormal cell growth, certain cellular survival factors are required to prevent inappropriate cell death. In an effort to elucidate the cellular mechanisms of chemoresistance in ovarian cancer, a number of novel cell survival proteins have been examined as potential determinants of chemosensitivity. Several such proteins are outlined below.

X-linked inhibitor of apoptosis protein

IAPs were first identified in baculovirus and the eukaryotic homologues were subsequently characterized [9]. Currently, the human IAP family includes X-linked inhibitor of apoptosis protein (Xiap), human inhibitor of apoptosis protein (Hiap) -1 and -2, neuronal apoptosis inhibitor protein (Naip), survivin and livin [10]. All IAPs contain one or more baculovirus inhibitor of apoptosis repeat (BIR) domains that block the substrate binding sites of target caspases (Figure 1). Xiap is a 55 kDa protein, which contains three BIR domains at the N-terminus. It requires no proteolytic processing for full function and has been shown to have high affinity for caspases. Xiap directly inhibits the initiator caspase-9 and execution caspase-3 and -7. Thus, Xiap attenuates both mitochondria/cytochrome c- and death receptor-mediated apoptosis [11]. Moreover, Xiap contains a C-terminus RING-Zinc finger domain with E3 ubiquitin ligase activity [12], which is believed to be involved in caspase degradation via the 26S proteasome. In addition, Xiap can undergo autoubiquitination, and Lys322 and Lys328 have recently been demonstrated to be the specific sites of ubiquitin ligation to Xiap [13]. However, the effects of Xiap autoubiquitination on its overall function remain unclear. In addition, the Xiap mRNA contains an Internal Ribosomal Entry Site (IRES) element [14], which allows Xiap to be translated in a cap-independent manner during cellular stress. In contrast, non-IRES containing mRNAs are translated exclusively in a 5' cap-dependent manner, a process that is often suspended during apoptosis due to the cleavage of transcription initiation factor 4G (eIF4G) by caspase 3. Recently, several cellular factors have been shown to bind Xiap and inhibit its activity, including Xiap Associated Factor 1 (XAF-1), Htr2A/Omi and Second Mitochondrial Activator of Caspases (Smac or DIABLO) [15–17]. It has been demonstrated that Smac is released from the mitochondria during dexamethasone-induced apoptosis in multiple myeloma cells [18], further supporting the role of Xiap as a central molecule in cell survival.

We previously reported that cisplatin down-regulates Xiap in chemosensitive, but not chemoresistant, ovarian cancer cells [8]. Moreover, Xiap is a determinant of chemoresistance, since downregulation of Xiap in chemoresistant cells rendered the cells sensitive to the cytotoxic actions of cisplatin, while overexpression of Xiap in chemosensitive cells caused a reversion to the chemoresistant phenotype [8,19].

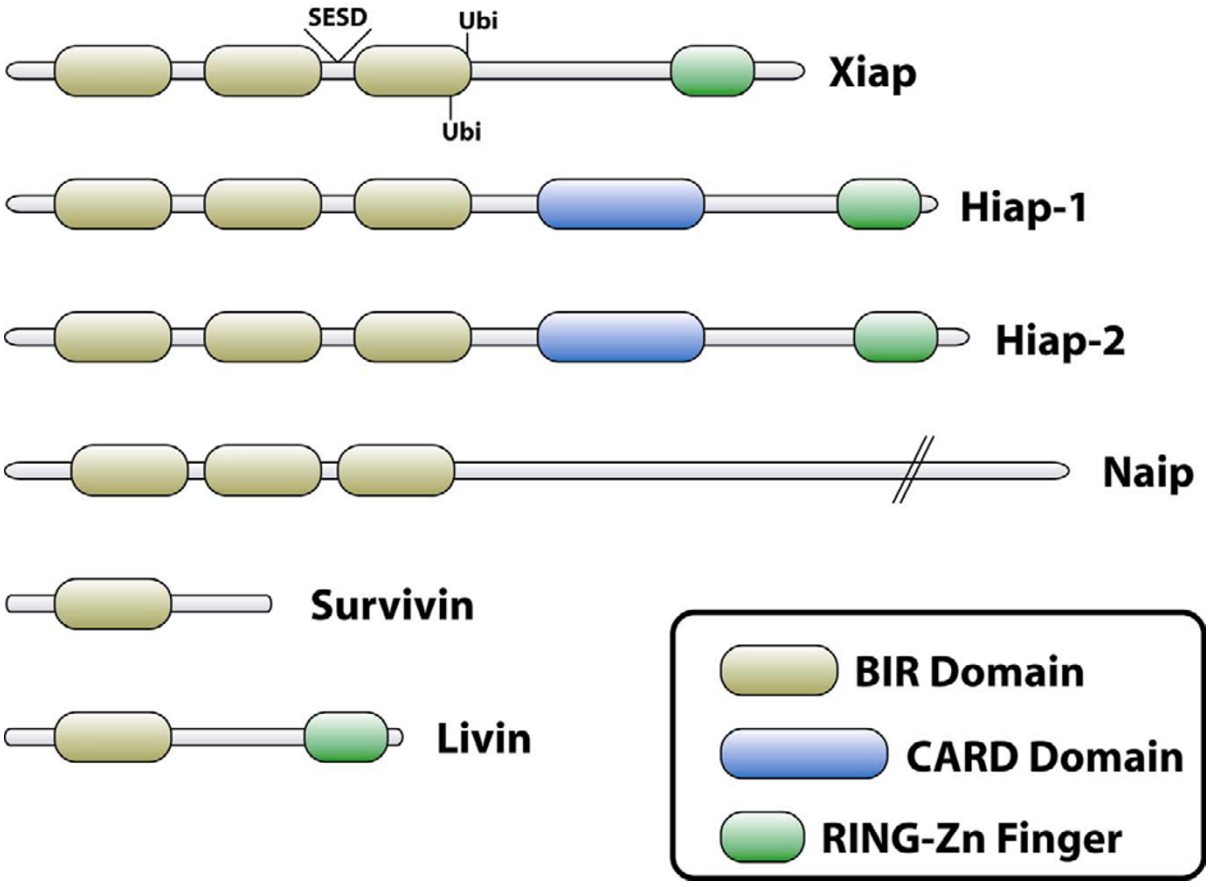

Figure 1
The Inhibitor of Apoptosis Proteins The human Inhibitor of Apoptosis Protein (IAP) family, originally characterized in baculovirus, currently consists of 6 members: X-Linked Inhibitor of Apoptosis Protein (Xiap), Human Inhibitor of Apoptosis Protein-1 and -2 (Hiap-1, -2), Neuronal Apoptosis Inhibitory Protein (Naip), survivin, and livin. Xiap is a 498-amino acid (~55 kDa) protein and is a potent inhibitor of caspases, including caspase-9, -7, and -3. Xiap contains three Baculoviral Inhibitory Repeat (BIR) domains, which are involved in direct inhibition of target caspases. Furthermore, Xiap contains a C-terminal RING-Zn Finger domain, which contains E3 Ubiquitin Ligase activity. In this regard, Xiap is known to ubiquitinate several target proteins, including caspase-3 and Smac/DIABLO. Xiap is also known to undergo autoubiqutination on Lys322 and Lys328. The role of Xiap's E3 activity in the regulation of apoptosis is unclear. Finally, Xiap is itself cleaved in a caspase-dependent manner during Fas-induced apoptosis. This cleavage occurs at the SESD sequence (amino acids 239–242), located between BIR domains 2 and 3, and results in the production of 2 ~30 kDa fragments of unknown function. Recent data from our laboratory suggests that Xiap is a determinant of cisplatin-mediated chemoresistance, and that the anti-apoptotic effects of Xiap are in part due to upregulation of the PI3K/Akt pathway and to suppression of p53-mediated cell death.

FLICE-like inhibitory protein confers TNFα resistance on ovarian cancer cells

Death receptors (DRs) are cell surface proteins belonging to the tumor necrosis factor (TNF) super-family. Recent studies suggest that these receptors or their downstream effectors play important role in regulating apoptosis, cellular growth and proliferation. The most widely studied DRs are Fas (also known as CD95), TNF receptor-1 and -2 (TNFR1 and TNFR2, respectively), and the TNF-related apoptosis inducing ligand (TRAIL) receptors, all of which are characterized by 1) cysteine-rich extracellular subdomains and 2) an intracellular region of about 80 amino

acids, termed the death domain (DD). The cysteine-rich extracellular subdomains are thought to adopt tertiary folds, required for intracellular clustering of DD motifs. The DD motif is essential for the transmission of cytotoxic signals and the induction of apoptotic cell death via recruitment of adapter proteins also containing a DD motif [e.g. Fas-associated death domain (FADD), TNFR-associated death domain (TRADD)] and formation of a receptor signaling complex [20].

Initiator caspases implicated in the DR-mediated apoptotic pathways (e.g. Caspase-8) are activated through interaction with the caspase-recruitment domain (CARD) motif of the DR-associating proteins [21]. Downstream execution caspases (e.g. caspase-3 and 7) are subsequently activated by initiator caspases. Two of the death receptor pathways, TNFα-TNFR1 and FasL-Fas, have been suggested to play an important role in chemoresistance in ovarian cancer cells.

Fas Ligand (FasL), a homotrimeric membrane protein, binds three Fas receptor molecules on the surface of the target cell, resulting in intracellular clustering of DDs and the recruitment of FADD to the receptor via homologous DD interactions [22]. The Fas-FADD complex recruits inactive caspase-8 (also called FLICE) by a homophilic interaction involving the death effector domains (DED) of FADD and FLICE. Upon recruitment by FADD, the pro-caspase-8 is cleaved to produce active caspase-8, which triggers activation of caspase-3 or -7. In human ovarian cancer cells, DNA damaging agents such as cisplatin can up-regulate Fas and FasL, resulting in activation of caspase-8 and -3 and induction of apoptosis [23]. We have shown that cisplatin is effective in inducing the expression of cell associated Fas and FasL, soluble FasL and apoptosis in two cisplatin-sensitive cell lines in a concentration- and time-dependent fashion [7]. In a chemoresistant variant, cisplatin failed to up-regulate FasL or induce apoptosis. Our results are supportive of the notion that dysregulation of the Fas/FasL system maybe an important determinant in cisplatin resistance in ovarian cancer cells.

Similar to the Fas/FasL pathway, binding of TNFα to TNFR1 results in receptor trimerization, death domain clustering, recruitment of TRADD and other proteins (e.g. FADD), cleavage and activation of pro-caspase-8 and the initiation of apoptosis. However, unlike the Fas-FasL system, binding of TNFα to TNFR1 is not sufficient to initiate apoptosis. Binding of TNFα to TNFR2 induces IκB phosphorylation and degradation and activates Nuclear Factor κB (NFκB) [24,25], which in turn regulates TNFα-induced apoptosis by inducing the expression of genes which modulate apoptotic pathways (e.g. IAPs and FLIP).

Fas-associated death domain-like interleukin-1β-converting enzyme (FLICE)-like inhibitory protein (FLIP) is a FADD-binding suppressor of apoptosis, which are present in two alternately spliced isoforms: long ($FLIP_L$) and short ($FLIP_S$) FLIP [26]. Both isoforms contain two death effector domains (DED), a structure resembling the N-terminal half of caspase-8 [27,28]. FLIP is recruited to the death-inducing signaling complex through FADD, thereby preventing caspase-8 recruitment and activation, and downstream apoptotic events [26,29] (see Figure 2).

We have recently demonstrated that TNFα induces NFκB-mediated $FLIP_s$ expression, which protects ovarian cancer cells from the cytotoxic action of the cytokine [30]. While TNFα alone was unable to induce apoptosis, it effectively induced apoptotic cell death in the presence of the protein synthesis inhibitor cycloheximide. These effects could be attenuated by sense $FLIP_s$ cDNA expression, suggesting that the resistance of ovarian cancer cells to the cytotoxic action of TNFα may be associated with the induction of $FLIP_s$ in response to the cytokine [30]. It is of interest to note that the role of FLIP in conferring resistance to cell surface receptor-mediated apoptosis is not confined to the actions of TNFα. TRAIL induces apoptosis in tumor cells via activation of death receptors DR4 and DR5 [31] and genotoxic agents (e.g. actinomycin D and ionizing radiation) sensitize TRAIL-induced cytotoxicity by decreasing intracellular levels of FLIP or increasing DR5 gene expression [32,33]. $FLIP_L$ also contributes to resistance to Fas-mediated apoptosis in MF-63 cells, and sensitization to Fas-mediated apoptosis by cisplatin [34]. In addition, since activation of the Fas/FasL system is an important mechanism of cisplatin-induced apoptosis in human ovarian cancer cells [7], the possibililty that FLIP is differentially regulated in chemoresistant ovarian cancer cells cannot be excluded.

The PI3K/AKT Cell Survival Pathway

Phosphoinositide 3-Kinases (PI3Ks) are heterodimers with separate regulatory (p85) and catalytic (p110) subunits. PI3Ks are generally activated upon recruitment of the inactive p85-p110 complex to receptor tyrosine kinases (RTKs), by interaction of the Src-homology 2 (SH2) domain of p85 with consensus phosphotyrosine residues at the receptors, in response to an appropriate physiologic cue (e.g. Growth factor stimulation) [35]. PI3K activation may be due to: 1) the close proximity of p110 to its lipid substrates in the membrane and 2) relief of the inhibitory effect of p85 on p110 kinase activity upon RTK-p85 interaction [36]. Direct binding of p110 to activated Ras proteins following growth factor stimulation further stimulates PI3K activity [37]. Class Ia PI3Ks are primarily involved in the phosphorylation of inositol-containing lipids, phosphatidylinositols (PtdIns), at the 3'-position of the inositol ring [38]. One of the reactions catalyzed by

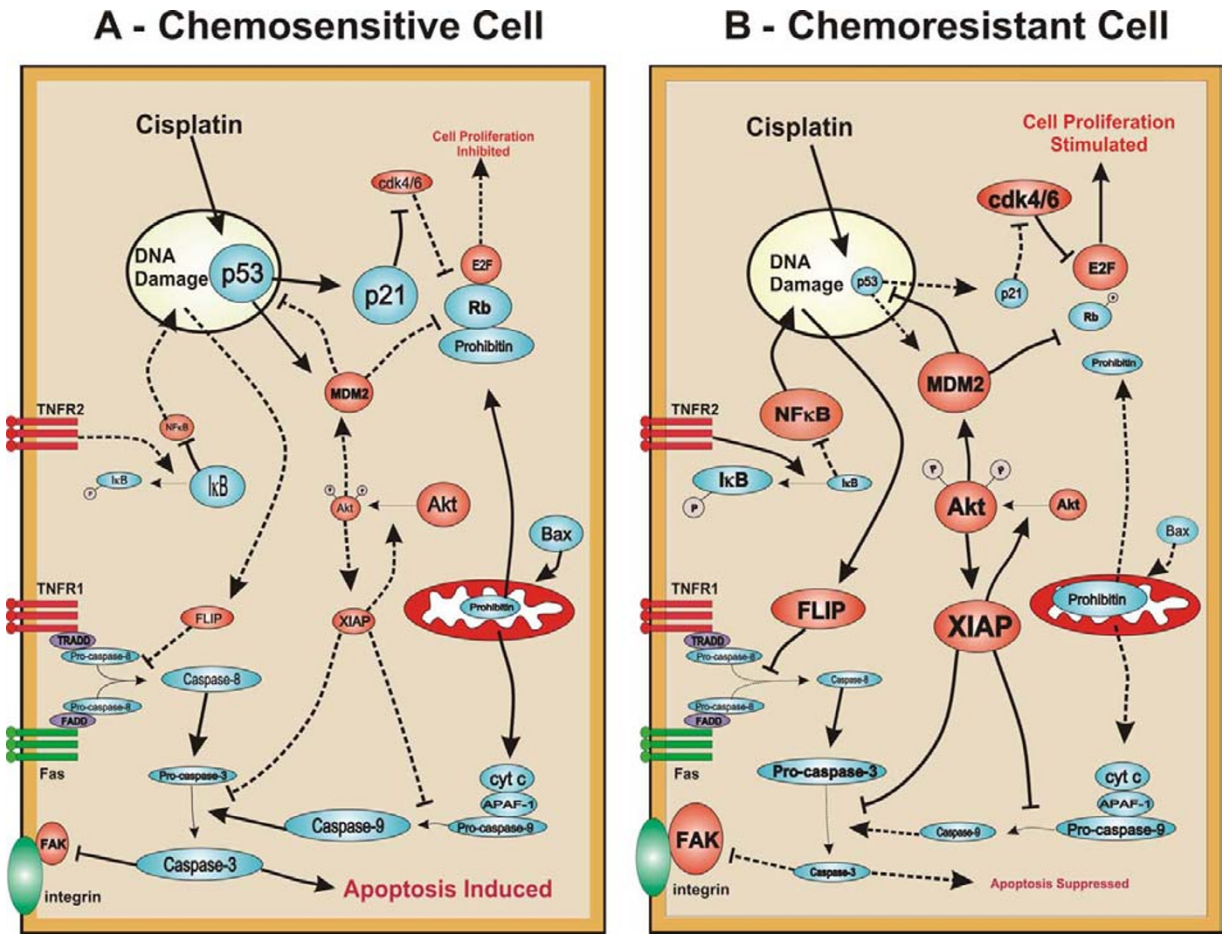

Figure 2

Hypothetical Model of Chemoresistance in Human Ovarian Cancer Cells. In a chemosensitive ovarian cancer cell (A), cisplatin increases p53 content, leading to upregulation of proteins promoting cell cycle arrest, such as p21, and of pro-apoptotic proteins such as Bax and Fas. This activates both the intrinsic (mitochondrial) and extrinsic (death-receptor) apoptotic pathways, the overall result of which is the activation of the execution caspase-3 (and -7, not shown). In these cells, cell survival mediators such as Xiap, Akt, and Flip (shown in red) are downregulated or are in their inactive state. Prohibitin may also play a role in inhibiting cell cycle progression through the Rb-E2F pathway by binding to Rb. Conversely, in a chemoresistant cell (B), increased p53 ubiquitination by MDM2 results in the maintenance of low levels of p53, despite the presence of cisplatin. Moreover, cisplatin fails to downregulate Xiap, thereby maintaining an active state of the PI3K/Akt pathway. In addition, binding of TNFR2 by TNFα leads to upregulation of FLIP through the NFκB pathway, thus inhibiting the pro-apoptotic actions of the cytokine through TNFR1. Overall, as a consequence of a failure to activate the caspase cascade in response to the chemotherapeutic agent, these cells have lost their capacity to undergo apoptosis, and thus became chemoresistant.

PI3K is the conversion of PtdIns-4,5-bisphosphate (PIP2) to PtdIns-3,4,5-trisphosphate (PIP3). Signaling proteins with pleckstrin homology (PH) domains accumulate at sites of PI3K activation by directly binding to PIP3. Of particular interest are AKT (a subfamily of the serine/threonine protein kinases), and phosphoinositide-dependent protein kinases (PDKs). Association of AKT and PDKs with PIP3 facilitates phosphorylation and subsequent activation of AKT by PDKs. Four sites (Ser^{124}, Thr^{308}, Thr^{450}, and Ser^{473}) are phosphorylated on AKT in vivo [39]. Whereas Ser^{124} and Thr^{450} appear to be phosphorylated under basal conditions [40], the Thr^{308} and Ser^{473}

residues are phosphorylated in response to a variety of extracellular stimuli, and are required for AKT activity.

Three AKT isoforms (AKT1, AKT2, and AKT3) have been identified, all of which share an N-terminal PH domain, a central kinase domain, and a serine/threonine-rich C-terminal region [40]. The overall homology among AKT1, AKT2 and AKT3 is more than 85%. AKT is the only downstream target of PI3K that has been associated with malignant transformation and its activation mediates a variety of cellular responses including cell growth, transformation, differentiation, motility, and cell survival. The intermediates of the PI3K/AKT survival pathway are often altered in human ovarian cancer and high levels of PI3K and AKTs have been linked to poor prognosis and chemoresistance [35]. Amplification of AKT2 has been demonstrated in breast, ovarian and pancreatic cancer [41–44]. Moreover, the gene that encodes the p110 subunit of PI3K is amplified in some cases of ovarian cancer, and the p85 subunit of PI3K is also targeted for mutation in human cancer [45]. A truncated p65 PI3K subunit (isolated from human tumor cell lines) causes constitutive activation of PI3K and cell transformation [46]. Activating mutations in the RTKs and loss of function of PTEN (phosphatase and tensin homology; a lipid phosphatase that inhibits PI3K/AKT signaling pathway by converting PIP3 to PIP2), in a wide array of human cancers furthermore supports the notion that PI3K/AKT pathway is involved in human cancer [47].

Recent evidence has demonstrated a role for the PI3K/Akt pathway in the resistance to a number of anti-tumor agents. We and others have demonstrated that cells expressing a constitutively active Akt2 are resistant to cisplatin [48,49]. Furthermore, a recent report has demonstrated that expression of a constitutively active PI3K catalytic subunit renders ovarian carcinoma cells resistant to paclitaxel, an effect that could be reversed by the PI3K inhibitor, LY294002 [50]. In this study, nude mice carrying ovarian cancer xenografts were treated with paclitaxel and LY294002. While both agents significantly reduced tumor burden alone (51 and 38%, respectively), they acted synergistically and reduced tumor burden by 80%, suggesting that inhibition of the PI3K/Akt pathway could potentially act as a potent adjuvant to traditional chemotherapies.

Focal Adhesion Kinase

Focal-Adhesion Kinase (FAK) is a 125 kDa, non-receptor protein tyrosine kinase, which is autophosphorylated on Tyr^{397} following association with integrin [51]. Activation of FAK is associated with suppressed apoptosis, an effect believed to result from activation of both the Ras/MAP Kinase pathway and the PI3K/Akt pathway [52,53]. It has been demonstrated that FAK suppresses detachment-induced apoptosis, and that inhibition of FAK activates caspases -3 and -8 and induces apoptosis [54]. Phosphorylation of Tyr^{397} promotes the binding of a number of SH2-containing proteins, including Src, PLCγ, Grb7, and PI3K [55–57]. In addition, phosphorylation of several C-terminal residues, including Tyr^{925}, promotes the activation of the Ras/MAPK pathway through activation of Grb2 [58]. The N-terminal of FAK is believed play a role in binding to the cytoplasmic tails of β integrin subunits [59]. The co-localization of FAK with integrins in focal adhesions appears to be necessary for cell adhesion-dependent activation of FAK. Both the catalytic activity and the major autophosphorylation site of FAK are required for the anti-apoptotic effects of FAK [60]. One recent study has demonstrated that the association of FAK with PI3K is a prerequisite for the inhibition of UV-induced apoptosis [61]. In addition, Tyr^{925}, which is implicated in the activation of the Ras/MAPK pathway, is also an important residue in the FAK-mediated inhibition of apoptosis [62]. Moreover, FAK overexpression enhances NFκB activity and increases expression of several members of the IAP family, including Xiap [63]. Finally, FAK contains a caspase-3 consensus D-X-X-D sequence, and has been shown to be a caspase-3 substrate [64], although the functional consequences of FAK cleavage are unclear. Nonetheless, FAK activity and stability appear to be closely related to a number of cell fate regulators and proper regulation of FAK appears to be required for the correct determination of cell fate.

Prohibitin

The prohibitin gene was originally cloned based on its ability to induce growth arrest at the G1/S phase of the cell cycle in mammalian fibroblasts and HeLa cells. It is located on human chromosome 17q21 close to the ovarian and breast carcinoma susceptibility gene BRCA1 [65], although no information currently exists on the role of prohibitin gene products in ovarian tumor ontogeny. Prohibitin mutations have been reported in a subset of sporadic breast cancers [65]. The prohibitin gene encodes a protein of 275 amino acids that is evolutionarily conserved in a wide variety of organisms. Prohibitin was found to share substantial homology with a *Drosophila melanogaster* gene product, Cc, of unknown function but vital for normal development and differentiation [66]. Flies homozygous for non-functional Cc alleles die during the larva to pupa metamorphosis. Within the parasitic worm, *Trypanosoma brucei*, prohibitin is upregulated during concanavalin A-induced apoptosis. The *Trypanosoma* prohibitin homologue appears to contain a putative death domain [67]. We have observed that prohibitin is up-regulated in normal ovarian cells undergoing apoptosis induced by gonadotropin releasing hormone (GnRH) agonist or gonadotropin withdrawal [68] and is primarily associated with the mitochondria in the ovarian cells. The

mechanism(s) by which prohibitin interacts with established cell death pathways to regulate apoptosis is unclear. Fusaro et al., (2002) demonstrated that stable over-expression of prohibitin in a human B cell line blocked apoptosis induced by the topoisomerase I inhibitor camptothecin and showed a protective role of prohibitin in breast cancer cell lines treated with the chemotherapeutic agent, suggesting that this protein may be a cell survival or anti-apoptotic factor, and plays an important role in determining the chemosensitivity of the cells [69]. Whether a similar anti-apoptotic mechanism for prohibitin is operational in the ovary remains to be determined. This interpretation could also account for the accumulation of prohibitin in the cytoplasmic or perinuclear region of epithelial cells in papillary serous ovarian carcinoma and endometrioid adenocarcinoma. Strong prohibitin immunoreactivity has been observed in these ovarian tumor cells. Moreover, prohibitin protein content correlates well with that of XIAP in these ovarian tumors and its level is higher than normal ovarian epithelium samples. The specific roles of prohibitin in ovarian cancer cell differentiation and mitochondrial metabolism await further investigation. However, it is possible that prohibitin may be important in the maintenance of mitochondrial integrity and that a loss of function might result in metabolic defects or apoptosis [70]. Finally, the observation that prohibitin translocates to the nucleus suggests it might also have a role in regulating cell cycle progression.

Cross-talk of Cell Survival and Death Pathways in Chemoresistance

While each of the aforementioned pathways may influence cell fate on its own, the cell must ultimately respond to a given stimuli (e.g. a chemotherapeutic agent) to either live or die. Thus, cross-talk between cell survival and cell death pathways ensures the cell receives an integrated signal which ultimately determines the fate of the cells (i.e. survival or death). This latter aspect has resulted in research activities that have significantly increased our current understanding of not only cell death/survival regulation, but also the molecular mechanisms of chemoresistance. Some of these activities are summarized hereafter.

Modulation of Death Receptor Signaling: Role of Xiap

Recent studies have demonstrated that Xiap may play a role in the control of both receptor-mediated cell survival and death signaling by directly inhibiting the principle effectors of apoptosis, downstream caspases [11,51]. Xiap plays a central role in promoting cell survival by inhibiting caspase-3 and -7 directly and modulating the Bax/cytochrome c pathway by inhibiting caspase-9. Structure-function analysis of Xiap has revealed that the region of Xiap containing the second BIR domain is necessary for inhibiting caspase-3 and -7 whereas the third BIR domain of Xiap is responsible for the inhibition of caspase-9 [71,72]. Xiap down-regulation following adenoviral Xiap antisense expression in ovarian cancer cell lines has been associated with onset of apoptosis, sensitization of chemoresistant cells to cisplatin, and detectable cleavage of caspase-3 [19,73]. In addition, Fas activation following FasL binding leads to initiation of the apoptotic process, which involves the activation of caspase-8 and caspase-3. By inhibiting caspase-3, Xiap attenuates Fas-mediated death signaling, which appears to be important in cisplatin-mediated apoptosis [7] (Figure 2).

The inability of TNFα to induce apoptosis upon binding to TNFR1 has been correlated to the induction of survival factors such as Xiap and FLIP [74,75]. Xiap does not appear to be an important determinant of resistance to the cytotoxic actions of the cytokine, as Xiap content in these cells is decreased by TNFα. We have shown that, in the presence of CHX, TNFα induces Xiap cleavage in these cancer cells [30]. Deveraux et al have shown that cleavage of Xiap results in different fragments with different specificity for caspases and even though the BIR2 domain of XIAP is required and sometimes sufficient for inhibition of caspase-3, the cleaved BIR2 fragment has reduced ability to inhibit caspase-3 and to suppress apoptosis [76]. These findings support the hypothesis that caspase-3-mediated processing of Xiap may somehow be involved in the execution of apoptosis in response to TNFα in ovarian cancer cells.

Previous studies have shown that sensitivity of melanoma cells to TRAIL-induced apoptosis is determined by level of expression of death receptor, TRAIL-R2 [77]. More importantly, there is a correlation between expression of this receptor and levels of activated caspase-3 in melanoma cells. However, despite high TRAIL-R2 expression and TRAIL-activated caspase-3 level, some melanoma cells were resistant to TRAIL-induced apoptosis. Inhibition of TRAIL-induced apoptosis by Xiap is the most possible explanation since over-expression of Xiap in TRAIL sensitive cell lines markedly suppressed TRAIL induced apoptosis whereas over-expression of Smac/DIABLO in TRAIL resistant melanoma cell lines sensitized the cells to TRAIL-induced apoptosis [77]. These results are consistent with the notion that Xiap suppresses TRAIL-induced apoptosis in these cells. Whether Xiap plays a similar role in the regulation of TRAIL-induced apoptosis, and thus the resistance of ovarian cancer cells to TRAIL, needs to be determined.

Modulation of Death Receptor Signaling: Role of FLIP

TNF-α, FasL and TRAIL are members of a growing family of cytokines which are cytotoxic to certain cancer cells

[21]. The sensitivity of cancer cells to cytokine-induced apoptosis is subject to complex regulatory processes, including the cell surface receptors and antiapoptotic proteins, such as FLIP, which can interfere with cytokine-mediated death pathways. TNFα is believed to play an important role in ovarian cancer biology and tumorigenesis, with concentrations significantly increased in ovarian cancer patients [78] and levels of TNFα expression positively correlated with tumor grade [79].

Our recent findings indicate that $FLIP_s$ plays a key role in conferring cellular resistance to the cytotoxic actions of TNFα in human ovarian cancer cells. While TNFα alone was unable to induce apoptosis, it effectively induced caspase-8 and -3 cleavage as well as apoptotic cell death when FLIP was downregulated by antisense expression [30]. Furthermore, up-regulation of $FLIP_s$ by $FLIP_s$ sense cDNA attenuated TNFα-induced cell death in the presence of the protein synthesis inhibitor cycloheximide [30]. In addition, challenge of ovarian cancer cells with TNFα resulted in a rapid increase in phosphorylated IκB content, which was temporally associated with NFκB translocation to the nucleus, and increased nuclear NFκB-binding activity and the expression of $FLIP_s$ but not of $FLIP_L$, all responses readily attenuated by dominant negative IκB. Our data also demonstrated that in the presence of CHX, TNFα induces Xiap cleavage in an ovarian cancer cell line, a process sensitive to the caspase inhibitors ZVAD and DEVD [30]. These findings are consistent with recent evidence showing that TNFα-induced $FLIP_s$ expression is mediated through activation of the NFκB signaling pathway and that FLIPs expression is an important determinant of resistance of human epithelial tumor cells to TNFα (Figure 2) [30,75,80,81]. They also support the contention that a caspase-3-mediated decrease in XIAP content may be involved in the execution of apoptosis in ovarian cancer cells in response to TNFα.

Xiap and FAK in Chemoresistance

As mentioned earlier, overexpression of FAK appears to promote the activation of NFκB and to enhance the expression of Xiap. Interestingly, FAK contains a consensus D-X-X-D caspase-3 cleavage site at amino acid 772. While caspase-3-mediated cleavage of FAK has been demonstrated in several studies, the exact consequences of this event are unclear. Cleavage of FAK at this site results in the separation of the C-terminal Focal Adhesion Targeting (FAT) sequence from the kinase domain. It is possible that this cleaved FAK may be ineffective in transmitting survival signals, or may actively promote cell cycle arrest and/or apoptosis [54].

We have recently investigated the role of FAK in cisplatin-mediated apoptosis in ovarian cancer cells [51]. Cisplatin induced FAK cleavage in a dose- and time-dependent manner in cultured cells. However, only those cells that detached from the culture surface following cisplatin challenge showed FAK cleavage. Moreover, the observed pattern of cisplatin-induced FAK cleavage could be recapitulated by the addition of recombinant active caspase-3 to whole-cell lysates. Overexpression of Xiap prevented cisplatin-mediated cleavage of FAK and rendered the cell resistant to the pro-apoptotic effects of the drug. These effects were dependent upon the presence of Xiap's BIR domains, suggesting that inhibition of caspases is an important determinant of Xiap-mediated protection of FAK and of cell viability. Finally, pharmacological inhibitors of caspase-3 could likewise inhibit cisplatin-induced cell detachment and FAK cleavage. Taken together, these results suggest that cisplatin-mediated apoptosis in ovarian cancer cells may involve cleavage of FAK, with subsequent disruption of cell adhesion. Since Xiap inhibits these processes and is a determinant of chemosensitivity in these cells, it is possible that Xiap-mediated chemoresistance may, in part, be due to maintenance of FAK integrity following cisplatin challenge (Figure 2).

A Central Role of p53 in Xiap- and Akt-mediated Chemoresistance

Although Xiap and the PI3K/Akt pathway are important cell survival factors, if and how they interact in the induction and maintenance of chemoresistance in human ovarian cancer is not known. We previously reported that cisplatin down-regulates Xiap in ovarian cancer cells which are chemosensitive but not chemoresistant [8]. Moreover, Xiap is a determinant of chemoresistance, since downregulation of Xiap in chemoresistant cells rendered the cells sensitive to the cytotoxic actions of cisplatin, while overexpression of Xiap in chemosensitive cells caused a reversion to the chemoresistant phenotype [8,19].

Several studies have linked the PI3K/Akt pathway to chemoresistance in ovarian cancer. Our laboratory has been particularly interested in the potential interactions between Xiap and the PI3K/Akt pathway. We have demonstrated that treatment of chemosensitive ovarian cancer cells with cisplatin decreases Xiap content, activates caspase-3 and -9, and induces Akt cleavage and apoptosis. However, these phenomenon are not observed in the chemoresistant counterpart [8,73]. Moreover, the ability of cisplatin to downregulate Xiap, and to induce Akt cleavage and apoptosis was attenuated by pretreatment with a cell-permeable caspase-3 inhibitor. These findings suggest that cisplatin-mediated apoptosis is dependent on caspase-3 activation, and involves modulation of the Akt cell survival pathway.

While failure of cisplatin to down-regulate Xiap is a hallmark of chemoresistance and Xiap overexpression in

chemosensitive ovarian cancer cells renders the cells resistant to cisplatin, the mechanistic underpinning for these phenomena is unclear. While several studies have established the inhibitory role of Xiap on caspase 3, 7 and 9, recent work has linked Xiap to a number of other signaling pathways, including the TGF-β/BMP and JNK pathways [82–85]. In addition, we have previously observed a co-localization between Xiap and phospho-Akt in human ovarian tumor sections and in nude mouse ovarian cancer xenografts (unpublished observations). Furthermore, Xiap and phospho-Akt staining was inversely correlated with apoptosis, as assayed by TUNEL staining. These preliminary findings suggest that there may be some relationship between Xiap and Akt, and intriguingly, that chemoresistance conferred by these cell survival intermediates may be related. Our recent work has demonstrated that Xiap overexpression in chemosensitive cells increases phospho-Akt content and attenuates cisplatin-induced apoptosis [73]. Total Akt (phosphorylated + non-phosphorylated) was not altered by Xiap, nor was the expression of the p85 subunit of PI3K, suggesting a direct influence of Xiap upon Akt activation rather than an up-regulation of Akt expression. Furthermore, Xiap-mediated chemoresistance could be attenuated by either the PI3K inhibitor LY294002 [73] or an adenoviral dominant-negative Akt [49], suggesting that activation of Akt is an important determinant of Xiap-mediated chemoresistance. Interestingly, our very recent results suggest that while cisplatin decreases Xiap content and induces apoptosis in chemosensitive ovarian cancer cells, the expression of a constitutively active Akt2 blocks both of these phenomena. Moreover, while cisplatin fails to down-regulate Xiap or induce apoptosis in chemoresistant cells, the expression of a dominant-negative Akt sensitizes cells to these effects of the chemotherapeutic agent. These results are significant because they suggest a feedback regulation of Xiap and Akt. While Xiap up-regulates Akt phosphorylation and requires Akt for its function, Akt protects Xiap from downregulation by cisplatin. Thus, it appears that there is an intricate, coordinated regulatory system at play between Xiap and the PI3K/Akt pathway, the end result of which leads to resistance to pharmacological doses of cisplatin.

In addition, we have been particularly interested in the possibility that aberrant regulation of the p53 tumor suppressor may be an important determinant of chemoresistance. *TP53* is a frequently mutated gene in malignant cells [4]. Its gene product (p53), a DNA-binding protein, functions as a sequence-specific transcription factor and is post-translationally modified by phosphorylation and ubiquitination [86]. p53 is usually sequestered in the cytoplasm as a complex with a p53-responsive gene product, Murine Double Minute-2 (MDM2), which limits its transactivational capacity. Moreover, MDM2 is a E3 ubiquitin ligase for p53, thus facilitating the degradation of p53 via the 26S proteasome [87].

When DNA is damaged, several upstream kinases in the cells, including ATM, ATR and MAP kinases (ERK, JNK, and p38), phosphorylate p53 on its N-terminal near the MDM2-binding site [88], thus decreasing the affinity of MDM2 for p53 and resulting in decreased ubiquitination and increased transactivational activity of p53. Free p53 enters the nucleus and binds to specific DNA sequences, either repressing or stimulating the expression of target genes. The role of p53 on cell fate following DNA damage depends on the duration and severity of the damage [89]. Short duration or less severe damage often results in the expression of proteins involved in cell cycle arrest and DNA repair, such as $p21^{WAF1/CIP1}$ and *GADD45*. p21 is required for p53-mediated suspension of the cell cycle at the G1 phase, while GADD45 is involved in DNA repair [90]. However, prolonged or severe DNA damage leads the upregulation of p53-responsive genes (e.g. *Bax*, *Fas*) and the induction of apoptosis through the mitochondrial death pathway [91]. Death receptor mediated apoptosis is also achieved by p53-mediated upregulation of Fas [90].

During apoptosis, MDM2 is cleaved, permitting the accumulation of p53 and thus the promotion of the apoptotic response. Xiap downregulation is associated with MDM2 cleavage, p53 stabilization and accumulation, and apoptosis in chemoresistant p53 wild-type ovarian cancer cells [19]. However, these phenomena are not observed in a chemoresistant p53 mutant cell line, unless wild-type p53 is first reconstituted, suggesting that p53 status may be an important determinant of Xiap-mediated chemoresistance. In addition, our recent studies demonstrate that while downregulation of Akt activity in p53 wild-type, chemoresistant ovarian cancer cells renders the cells sensitive to cisplatin, this effect is not observed in p53 mutant, chemoresistant cells. Again, the phenomenon is restored by expression of exogenous wild-type p53. Addition of the specific p53 inhibitor Pifithrin-α-hydrobromide (PFT) to ovarian cancer cell culture reverses the dominant-negative Akt-mediated sensitization of the chemoresistant, p53 wild-type cells, and the chemoresistant p53 mutant in which wild-type p53 was reconstituted [49].

Cisplatin up-regulates p53 in a number of systems [6,92]. However, the precise mechanisms that give rise to this phenomemon are not clear. We have recently demonstrated that while cisplatin increases p53 content in chemosensitive, p53 wild-type ovarian cancer cells, it fails to do so in a chemoresistant, p53 wild-type variant [49]. However, cisplatin is capable of upregulating p53 and inducing apoptosis in these cells following expression of dominant-negative Akt, suggesting that the cisplatin-

mediated upregulation of p53 is opposed by Akt. Interestingly, MDM2 has been shown to be a target of phosphorylation by Akt [93–96]. These studies have established that phosphorylation by Akt activates MDM2, thereby maintaining p53 levels low. However, whether this process contributes to the failure to upregulate p53 following cisplatin challenge in chemoresistant cells is not known. Taken together, these results suggest that p53 status is an important determinant of Xiap- and Akt-mediated chemoresistance (Figure 2).

Interaction of Prohibitin with Retinoblastoma (Rb) Protein

The prohibitin gene is a potential tumor suppressor that has been linked to human cancer. Sato et al (1992, 1993) identified four mutations in 23 sporadic breast cancer patients and provided the first evidence for the tumor suppressive nature of prohibitin in breast cancer tumorigenesis [65,97]. More recently, Jupe et al. (2001) identified two prohibitin alleles that correlate with the predisposition to breast cancer tumorigenesis in a clinical study involving 205 patients [98]. Although the RNA encoding the 3' UTR of the prohibitin gene has been reported to suppress cell proliferation by blocking G1/S transition of the cell cycle [99], the mechanism by which this occurs is unclear. The *Rb* gene was the first tumor suppressor to be cloned, but the mechanism behind its role in tumor suppression remains unclear. Accumulating evidence indicates a complex role for Rb in cell proliferation, differentiation and survival. The retinoblastoma gene was initially identified as a genetic locus associated with the development of an inherited eye tumor [100,101]. The realization that it was a loss of function of Rb that was associated with disease established the tumor suppressor paradigm [102]. Subsequent work identified the E2F transcription factor activity as a key target for the growth suppressing action of the Rb protein [103]. Additional studies demonstrated that Rb function requires interaction with E2F and phosphorylation; a mechanism that is mediated by D-type cyclin-dependent kinases [104–106]. D cyclin/cdk4 activity is induced by growth stimulation, thus initiating the cascade of events that leads to E2F accumulation and S-phase entry [107]. Wang et al. reported that prohibitin physically interacts with Retinoblastoma (Rb) protein and regulates E2F function, a response correlated with its growth suppressive activity [108,109] (Figure 2). Although the molecular mechanism by which prohibitin-mediated E2F repression is not fully understood, the recruitment of the histone deacetylase HDAC1 for transcriptional repression of the E2F family and the involvement of co-repressors such as NcoR appears necessary [98]. Evidence also exists that prohibitin can also act independently of Rb. Some E2F-responsive promoters appear to be unaffected by the recruitment of HDAC [110], suggesting alternative mechanisms involving other transcriptional repressors (e.g. Brg-1 and Brm) may be involved [111–113]. In this context, Wang et al (2002) demonstrated that prohibitin requires Brg-1 and Brm for transcription repression of E2F-1 [114]. The recruitment of Brg-1 and Brm by prohibitin is independent of Rb, while the transcriptional repression mediated by prohibitin/Brg-1/Brm requires Rb, and is reconstituted when Rb is co-transfected in Rb-deficient cells. Whether similar regulatory mechanisms involving prohibitin exist in the ovary is currently under investigation.

Conclusions and Future Directions

Chemoresistance is a therapeutic problem that severely limits successful treatment outcomes for many human cancers. This is particularly true of ovarian cancer, where the development of resistance is a common occurrence. The past few years have seen an enormous growth in our understanding of the mechanisms that regulate drug-induced apoptosis, and thus influence chemosensitivity. However, it is important to remember that no biochemical pathway stands on its' own. While these pathways have previously been considered disparate, recent studies have shown complex interactions between them in cell fate regulation. Xiap, for example, was originally characterized according to its' ability to inhibit caspases. However, recent data suggests that this anti-apoptotic protein interacts with a number of fundamental cell survival pathways, such as the PI3K/Akt pathway. Similarly, the p53 tumor suppressor, while critically important in the mediation of apoptosis triggered by chemotherapeutic agents, has now been shown to be itself regulated by anti-apoptotic proteins such as MDM2 and Akt. Ultimately, the success of chemotherapy depends on our understanding of the integration of these pathways, with the issuance of a proper life or death signal. While the recent discovery of these biochemical interactions is intriguing, it must be noted that our understanding of the precise mechanisms by which these interactions occur is incomplete. For instance, while Akt appears to attenuate the effects of cisplatin on p53 (i.e. upregulation) and on Xiap (i.e. downregulation), the mechanisms involved are unclear. One intriguing possibility is that specific post-translational modifications critical for the response to cisplatin may be altered in chemoresistant cells. For instance, under specific cellular conditions, both p53 and Xiap are ubiquitinated and degraded in the 26S proteasome. It is possible, therefore, that Akt may be influencing the stability of either or both of these proteins via altered ubiquitination pathways. These types of specific mechanistic questions will drive the future of research in this area. In addition, the development of advanced molecular techniques will be crucial for the success of this endeavour. The next few years will likely be critical in determining the precise mechanisms controlling the onset and maintenance of

chemoresistance, and will likely suggest several new methods for overcoming this enormous clinical problem.

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