Molecular mechanisms of apoptosis and chemosensitivity to platinum and paclitaxel in ovarian cancer: biological data and clinical implications

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Summary

Apoptosis is a genetically regulated biological process that plays a major role in chemotherapy-induced tumor cell killing.

It may be triggered by two major intracellular signaling cascades, the mitochondrial pathway and the death receptor pathway, both leading to caspase activation and cleavage of specific cellular substrates. The p53 gene is involved in the regulation of apoptosis. Caspase activation following wild-type p53 induction is associated with the release of the apoptogenic factors cytochrome c and Smac/DIABLO from the mitochondria, that is in turn controlled by the pro-apoptotic and anti-apoptotic Bcl-2 family proteins. In ovarian cancer p53 status is a strong predictor of response to platinum-based chemotherapy. Patients whose tumors have p53 mutations experience a lower chance of achieving a complete response following platinum-based regimens when compared to patients without p53 mutations. Conversely, experimental and clinical data seem to show that paclitaxel enhances apoptosis through a p53-independent pathway, that probably involves the Bax gene. Whereas patients with wild-type p53 tumors have a good chance to respond to platinum, patients with mutant p53 tumors may have a clinical benefit from the addition of paclitaxel to platinum-based chemotherapy. Therefore determining p53 status can be useful in predicting therapeutic response to specific drugs.

Moreover the understanding of cellular mechanisms regulating apoptosis might offer a strong rationale for the combination of chemotherapy with other biological treatments.

Key words: Apoptosis; Ovarian Cancer; p53; bcl-2; Chemotherapy.

Introduction

In several types of carcinomas response to chemotherapy involves activation of apoptosis, which represents a morphologically and biochemically distinct form of cell death, characterized by surface blebbing, cytoplasmic contraction, chromatin condensation, internucleosomal cleavage of genomic DNA, and packaging of cellular components within membranes prior to their budding from the cell as apoptotic bodies [1-5]. The activation of aspartate-specific cysteine proteases, termed caspases, plays a major role in apoptosis. Caspases, which are present in most cells as inactive proforms and are activated by proteolytic processing, can be divided into initiator caspases [caspases 8, 9, and 10] and effector caspases [caspases 3, 6, and 7]. The sequential cleavage of one caspase by another one creates a cascade of proteolytic activity, ultimately leading to cell death [6].

Phosphatidylserine externalization and binding by annexin-V is an early membrane marker of apoptosis [7]. Phosphatidylserine translocation is followed rapidly by oligo-nucleosomal DNA fragmentation, after which cell and nuclear membrane leakage occurs [7, 8]. The addition of specific caspase inhibitors blocks phosphatidylserine externalization and DNA fragmentation,indicating that these events are down-stream from caspase activation [7].

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This report focuses on molecular mechanisms of apoptosis and implications for sensitivity and resistance to chemotherapeutic agents in ovarian cancer.

Molecular mechanisms of apoptosis

The p53 gene, that encodes a nuclear phosphoprotein normally acting as a guardian of the integrity of the genome, plays a major role in the regulation of apoptosis [9]. After DNA damage the increased levels of the wild-type p53 may trigger a complex series of reactions leading to either cell cycle arrest at G1/S check-point or alternatively to apoptosis [4, 10-12]. These reactions are mediated through transcriptional regulation of several genes, such as those encoding the cyclin-dependent kinase inhibitor p21/WAF1/CIP1 and the pro-apoptotic and anti-apoptotic members of the bcl-2 family (Table 1). Phosphatidylinositol 3-kinase (PI3K) and its downstream targets serine/threonine kinase AKT1 and AKT2 are required for the expression of p21/WAF1/CIP1 induced by cisplatin (CDDP) and paclitaxel (TAX) in human ovarian carcinoma cell lines expressing wild-type p53, but not in those lacking functional p53 [13]. Therefore the PI3K/AKT signal transduction pathway is involved in p53-mediated cell cycle regulation.

Two major intracellular apoptosis signaling cascades have been characterized, the mitochondrial pathway and the death receptor pathway [6, 14].

Caspase activation following wild-type p53 induction is associated with the release of the apoptogenic factors cy-

Table 1. — *Bc1-2 oncogene family and apoptosis.*

Pro-apoptotic members	Anti-apoptotic members
Bax	bcl-2
bcl-Xs	bcl-Xl
Bak	bcl-w
Bok	Mcl-1
Bid	BOO/DIVA
Bad	Al/Bfl-1
Noxa	NR-13
PUMA	

tochrome c and Smac/DIABLO from the mitochondria [15]. Such release is regulated by the pro-apoptotic and anti-apoptotic bcl-2 family proteins, which either induce or prevent the permeabilization of the outer mitochondrial membrane [6, 15-17].

Cytochrome c catalyzes the oligomerization of apoptotic protease activating factor-1 and the activation of procaspase-9, thereby cleaving and activating caspase-3 and caspase-7 which execute the cell death program [4, 17]. Smac/DIABLO is a mitochondrial protein that enhances caspase activation, by neutralizing one or more members of inhibitors of the apoptosis protein (IAP) family such as the X-linked inhibitor of apoptosis protein (XIAP) [18-22].

IAPs prevent cell death by inhibiting caspases.

Besides internal signals which arise from DNA damage, external signals may trigger apoptosis through binding to cell surface membrane death receptors (DRs) [5, 23-26]. These receptors are the products of the tumor necrosis factor (TNF) receptor gene superfamily, which includes Fas, DR3, DR4, DR5 and DR6; the binding of specific ligands, such as Fas-ligand (Fas-L) and TNF-related apoptosis-inducing ligand (TRAIL), to DRs induces their clustering and triggers a series of reactions leading to proteolytic activation of caspase 8, which in turn cleaves and activates caspase 3. However recent data show that mitocondria and associated Bax, bcl-2 and bcl-Xl proteins may be involved in DR-induced apoptosis by modulating the release of Smac/DIABLO [26- 28]. In human melanoma cell lines TRAIL was found to cause release of Smac/DIABLO from mitochondria and to down-regulate XIAP levels [29]. Other experimental studies detected that in HeLa cells exposed to TRAIL caspase-8 activation rapidly occurred in association with bid cleavage, cytochrome-c release, caspase-3 activation, and DNA fragmentation, whereas the addition of a caspase-8 inhibitor prevented caspase-3 activation and apoptotic cell death [25].

Cells expressing Fas can undergo apoptosis upon exposure to either Fas-L [30] or an agonistic Fas antibody [31]. The presence of Fas mutations may represent a mechanism of resistance to apoptosis signaling [32]. Shedding of Fas-L from the cell surface has been observed in tumor cells, and may provide a mechanism for partial protection from apoptosis [33, 34]. The metalloproteinase (MMP)-7 has been found to catalyse this process [35]. DNA-damaging drugs, such as doxorubicin, kill cancer cells, at least in part, by up-regulating Fas-L, and MMP inhibitors can potentiate the activity of chemotherapeutic drugs by blocking the proteolytic cleavage of Fas-L [36]. Soluble Fas (sFas) is produced by alternative splicing of Fas mRNA encoding a soluble form of the Fas protein that lacks the transmembrane domain; sFas binds to and neutralizes Fas-L, thus antagonizing Fas/Fas-L- mediated apoptosis [37].

In conclusion, both the mitochondrial pathway and the death receptor pathway lead to caspase activation and cleavage of specific cellular substrates, resulting in the morphological and biochemical changes associated with the apoptotic phenotype [4].

The ubiquitin-proteasome degradation pathway is the main post-transcriptional mechanism that controls the levels of many short-lived proteins involved in regulating cell cycle progression, DNA transcription, DNA repair, and apoptosis [38]. Proteins are usually targeted for proteasome-mediated degradation by polyubiquitinylation, the covalent addition of multiple units of the 76 aminoacid protein Ub which are bound to 1-amino groups of lysine residues in the substrate [39]. Polyubiquitinylated proteins are degraded by the 26S proteasome, a large, ATP-dependent multicatalytic protease complex, which also regenerates monomeric Ub. It has been recently detected that proteasomes catalyse key events in the activation of the transcription nuclear factor-kappaB (NF-kappaB), which inhibits the apoptotic response to chemotherapy and radiotherapy [40, 41]. Moreover interleukin 1-alpha, an important regulatory cytokine expressed autonomously by several malignancies, can enhance the activation of NF-kappaB [42]. The antiapoptotic effect of NF-kappaB is probably mediated through the induction of IAP family proteins [18].

Early experimental and clinical studies revealed that proteasome inhibitors are able to delay cancer progression and to enhance the apoptotic response to chemotherapeutic agents, and therefore might represent a novel approach to cancer treatment [41, 43-46].

The proteasome inhibitor PS-341 has recently entered multiple phase I-II clinical trials for the treatment of multiple myeloma, chronic lymphocytic leukemia, and a variety of solid tumors [44].

The inactivation of proteasome function can inhibit inducible NF-kappaB activation, thereby increasing the apoptotic cell death following chemotherapy and radiotherapy [40, 46]. For instance the pretreatment of human colorectal cancer cells with PS-341 before exposure to SN-38, the active metabolite of the topoisomerase-1 inhibitor irinotecan, blocked the NF-kappaB activation and resulted in a significantly higher growth inhibition compared with treatment with PS-341 alone or SN-38 alone [40]. Similarly the inhibition of NF-kappaB activation increases radiation-induced apoptosis in colorectal cancer cells in vitro and in vivo [46].

Apoptosis and response to chemotherapy in ovarian cancer

Experimental studies on monolayers of ovarian cancer cell lines and primary ovarian cancer cells obtained from ascites showed that tumor cell death in response to the commonly used drugs, such as CDDP, cyclophosphamide (CTX) or TAX, involves the induction of apoptosis rather than simply necrosis [47].

As for genetic changes involved in cell cycle and apoptosis regulation, p53 mutations and/or p53 protein overexpression have been detected in 20-79% of ovarian cancers [48-61]. p53 alteration rate is higher in advanced than in early stages of the disease.

In vitro data showed that the presence of altered p53 in resistant ovarian cancer cells might be involved in the relative failure of CDDP-induced apoptosis [62-64]. On the other hand, the introduction of wild-type p53 through adenovirus gene transfer significantly sensitized the human ovarian A2780/CP tumor cells, carrying mutant p53, to CDDP cytotoxicity [65].

A significant correlation has been detected between p53 status in tumor samples collected during initial surgery and response to CDDP- or carboplatin (CBDCA)-based chemotherapy in patients with ovarian cancer [54, 55, 61, 66, 67]. Patients whose tumors had p53 mutations experienced a lower chance to achieve a complete response following platinum-based regimens when compared to patients without p53 mutations. Therefore, the loss of p53 function and resistance to apoptosis induction can represent major mechanisms of platinum chemoresistance.

As for prognosis of ovarian cancer patients, in some series p53 status did not correlate with survival [48, 50], whereas other authors found that the clinical outcome was poorer in patients with p53 alterations [51, 55, 61]. In a study performed on 178 ovarian cancer patients treated with platinum-based chemotherapy, time to progression and overall survival were significantly shortened in patients with p53 mutations compared with those with wild-type p53 ($p = 0.029$ and $p = 0.014$, respectively) [61]. However at multivariate analysis p53 status was not found to be an independent prognostic factor.

A recent study reported that CDDP decreased XIAP levels and enhanced AKT cleavage and apoptosis in chemosensitive, but not in chemoresistant, ovarian cancer cells [68]. Adenoviral sense XIAP cDNA expression increased XIAP protein levels, increased AKT phosphorylation, and decreased CDDP-induced apoptosis. Infact, after its activation by phosphorylation, AKT can phosphorylate and inactivate several proteins involved in apoptosis including Bad, a pro-apoptotic member of the bcl-2 family, and caspase 9 [5]. However, in the presence of a PI3K inhibitor XIAP overexpression failed to induce AKT phosphorylation and to block CDDP-induced apoptosis [68]. Therefore XIAP might prevent apoptosis through a PI3K-dependent inhibition of the caspase cascade. These data are in agreement with those of Li *et al.* [69] who found that antisense down-regulation of XIAP induced apoptosis in CDDP-sensitive and, to a lesser extent, in CDDP-resistant human ovarian cancer cell lines. XIAP might represent a novel target for gene therapy of ovarian cancer, and the use of XIAP antisense alone or in combination with wild-type p53 sense might offer a new approach for the treatment of chemoresistant disease [70].

Little is known about the expression and the clinical relevance of p53 downstream genes in ovarian cancer. Positive immunostaining for bcl-2, bcl-Xl, and Bax has been reported, respectively, in 19-57% [51, 57, 71], 62% [72], and 60-66% [72, 73] of cases of ovarian carcinomas. Significantly less immunoreactive bcl-2 protein has been detected in malignant ovarian serous tumors compared to their benign counterparts [74]. Most authors reported that the presence of bcl-2 in ovarian cancer is a favorable prognostic indicator [71, 74, 75], and others observed that Bax expression is associated with a good clinical outcome [72, 76]. For instance Schuyer *et al.* (76) found that, according to the Cox model, Bax expression is related to a better progression-free survival and overall survival ($p = 0.05$ and $p = 0.03$, respectively), and that patients who simultaneously express Bax and bcl-2 have a longer progression-free survival and overall survival compared to patients whose tumors do not express bcl-2 ($p = 0.05$ and $p = 0.015$, respectively). However, in the study of Mano *et al.* [57], including 66 patients with advanced ovarian cancer, there was an inverse relationship between bcl-2 staining and response to chemotherapy, expecially in patients with serous and endometrioid carcinomas.

In vitro investigation showed that bcl-Xl exerts an anti-apoptotic effect on ovarian cancer cell lines exposed to CDDP and TAX [77]. Infact the A2780 cells expressing low levels of endogenous bcl-Xl are chemosensitive, while the SKOV3 cells having elevated amounts of the protein encoded by this oncogene are chemoresistant. After tranfection with bcl-Xl containing plasmids, A2780 cells become highly resistant to CDDP and TAX.

Xiang *et al.* [78] detected that overexpression of Bax both directly induced apoptosis and enhanced chemotherapy-induced cytotoxity in established ovarian cancer cell lines as well as in patient-derived primary ovarian carcinoma cells.

Experimental data provided evidence that TAX enhances apoptosis through a p53-independent pathway [79-83)]. Loss of p53 function in A2780 human ovarian cancer cells conferred increased resistance to several DNA-damaging agents, but not to TAX or camptothecin [80]. In the ovarian cancer cell lines SKOV3 and KP, which have a homozygous deletion of the p53 gene, wild-type p53 gene-transduction markedly enhanced the sensitivity to CDDP, but not to TAX [82].

Morphologically a sustained block of mitosis seems to be required for TAX-induced apoptosis, even if the events occurring between mitotic arrest and the subsequent onset of apoptosis are still unclear. Chadebech *et al.* [84] detected that TAX-induced microtubule damage inhibits proteasome-dependent degradation of cyclin B, thereby resulting in a sustained activation of cyclin B/cdc2 kinase and a cell cycle arrest in mitosis with a G2/M DNA content. ErbB2 overexpression, which confers resistance to certain chemotherapeutic agents such as TAX, leads to deregulation of the G2/M cell cycle check-point, thus blocking TAX-induced apoptosis [85]. Trastuzumab can effectively sensitize ErbB2-overexpressing breast cancer cells to TAX by reversing the anti-apoptotic function of ErbB2.

The IGROV-1 ovarian cancer cell line, containing wild-type p53, and its CDDP-resistant p53 mutant

subline IGROV-1/Pt1 shows quite different time course of taxane-induced cell death [86]. Apoptosis is an early event consequent to a transient mitotic arrest in the former, whereas the cell death of the latter is a somewhat slow and delayed event, following mitotic arrest and appearance of hyperploid cells.

Bax might be involved in TAX-induced apoptosis. Strobel *et al.* [79] transfected the SW626 human ovarian cancer cell line, which lacks functional p53, with a cDNA encoding for murine Bax, and detected that the cytotoxicity of TAX was significantly enhanced and was associated with enhanced apoptosis in Bax-transfectants compared with control clones. Thereferore Bax might stimulate TAX-induced apoptosis through a p53-independent pathway. Several clinical observations confirmed that sensitivity to TAX-based chemotherapy is independent of p53 status [56, 58-60, 87], and some early clinical data seem to suggest that it is related to Bax expression [73].

Lavarino *et al.* [87] found that all but one of the ten ovarian cancer patients who had positive immunocytochemistry for p53 accumulation achieved a complete pathological or clinical response to a combination chemotherapy with TAX and CBDCA. Smith-Sorensen et al. [56] detected p53 mutations in 73% of tumor samples from 45 ovarian cancer patients randomized to receive TAX plus CDDP or CTX plus CDDP. They found that, among patients with p53 alterations, relapse-free survival was significantly longer for the TAX plus CDDP group compared with the CTX plus CDDP group ($p = 0.002$) and that, among patients treated with the TAX-based regimen, there was no relationship between p53 status and prognosis. A retrospective investigation on 43 patients with advanced ovarian cancer treated with TAX-based chemotherapy showed that p53 status was predictive of neither chemoresistance nor disease-free and overall survival [59]. In a multicentric Italian study performed on 38 patients with advanced disease treated with TAX plus CBDCA with or without epidoxorubicin, there was no significant difference in complete response rates and survival rates between patients whose tumors overexpressed p53 protein and patients whose tumors did not [58].

Another multicentric Italian study [60] assessed p53 status by genetic and immunohistochemical analysis in tumor specimens collected at the time of initial surgery from 48 advanced ovarian cancer patients who subsequently received TAX-plus platinum-based chemotherapy. The overall response rates and the complete response rates were significantly higher among patients with mutant p53 tumors compared with those with wild-type p53 tumors ($p = 0.008$). Therefore the pattern of response to TAX-based chemotherapy seems to be quite different with respect to that associated with platinum-based regimens.

Very interesting data emerged from the study of Tai *et al.* [73] who investigated the relationship between Bax protein expression and clinical outcome in 45 ovarian cancer patients treated with a first-line regimen including TAX plus a platinum analogue. Patients whose tumors expressed high levels of Bax experienced a higher complete response rate to chemotherapy (100% vs 57%, $p = 0.036$) and a longer median disease-free survival after a median follow-up of 1.9 years (not reached vs 1.1 years, $p = 0.0061$) when compared to low-Bax expressors.

Other experimental data revealed the involvement of AKT gene products [88] as well as caspases in TAX-induced apoptosis [89, 90]. In fact ovarian cancer cells either overexpressing constitutively active AKT1 or containing AKT2 amplification are highly resistant to TAX compared to cancer cells expressing low AKT levels [88]. As previously reported, AKT gene products can inactivate proteins involved in the apoptotic process [5]. In breast cancer cell lines TAX-induced apoptosis is blocked by a broad-spectrum caspase inhibitor and is increased by the cyclin-dependent kinase inhibitor flavopiridol which is able to enhance caspase activation [89, 90].

Twelve chemoresistant ovarian cancer cell lines were treated with each chemotherapeutic drug alone, CDDP, doxorubicin or TAX, TRAIL alone, or combination [91]. The majority of chemoresistant cells were also resistant to TRAIL alone, whereas the combination of TRAIL and chemotherapy resulted in a significant growth inhibition associated with a significant increase in the fraction of apoptotic cells and in caspase activation. Therefore this combined treatment overcomes the resistance by triggering caspase-mediated apoptosis. Other in vitro investigations confirmed the ability of TRAIL to boost the apoptotic response to TAX and CDDP in ovarian cancer cell lines [92].

Very few data are currently available on the expression and the role of the Fas/Fas-L system in ovarian cancer [71, 93-97]. This death receptor pathway seems to be involved in ovarian cancer cell apoptosis in vitro. Exposure to anti-Fas monoclonal antibodies caused apoptosis in the two ovarian cancer cell lines HEY and Caov-3 [93]. Fas-associated phosphatase-1(FAP-1) is a protein-tyrosine phosphatase that binds to the cytosolic Fas tail, presumably regulating Fas-induced apoptosis [97]. Elevated FAP-1 levels were found in the Fas-resistant ovarian cancer cell lines OVCAR-3, FR and SK-OV-3, but not in the Fas-sensitive ovarian cancer cell lines HEY and BG-1. CDDP-induced apoptosis in ovarian cancer cells seems to be partly due to upregulation of the Fas/Fas-L system [70)]. Munakata et al. [71] detected Fas-L expression in 3% of 36 benign ovarian tumors, in 36% of 33 ovarian tumors of low malignant potential and in 67% of 63 ovarian carcinomas. Serum sFas levels are significantly higher in ovarian cancer patients than in healthy control females, and are related to tumor stage [95, 96]. Elevated pretreatment serum sFas seems to be an independent poor prognostic factor for disease-free survival and overall survival in patients with this malignancy.

Conclusions

The induction of apoptosis is the main cause of cell death in ovarian cancer following chemotherapy. This process may be triggered by two major intracellular

signaling cascades, the mitochondrial pathway and the death receptor pathway, both leading to caspase activation and cleavage of specific cellular substrates. The understanding of cellular mechanisms regulating the apoptosis might shed light on the biochemical pathways involved in the response to chemotherapeutic agents, and might offer a strong rationale for the combination of chemotherapy with other biological treatments.

The loss of p53 function represents a major mechanism of platinum-resistance in ovarian cancer. Conversely TAX seems to enhance apoptosis through a p53-independent pathway, which probably involves the Bax gene. Patients with wild-type p53 tumors have a good chance of responding to platinum, whereas patients with mutant p53 tumors may have a clinical benefit from the addition of TAX to platinum-based chemotherapy. Therefore determining p53 status can be useful for a rationale planning of chemotherapy in ovarian cancer patients. Moreover, the combination of chemotherapeutic agents with gene therapy (recombinant adenovirus carrying wild-type p53 or Bax gene), or with ligands for DRs (TRAIL or anti-Fas monoclonal antibodies), or with proteasome inhibitors will represent an interesting field of clinical research aimed at increasing the apoptotic response to chemotherapy in ovarian cancer.

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