Towards an understanding of the biological basis of response to cisplatin-based chemotherapy in germ-cell tumors

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Chemotherapy is far more successful in young male patients with germ-cell tumors than in adults suffering from almost any other solid tumor. Various attempts have been made to understand the sensitivity of these tumors towards cisplatin-based chemotherapy; however, to date no explanation has been generally accepted. Recent data underline the need to seek further explanations, other than the previously postulated high intrinsic level of wild-type P53 protein, for the exquisite curability of germ-cell tumors. In this regard, the DNA repair pathways, in particular the DNA mismatch repair and nucleotide excision repair pathways, have received attention. This review summarizes the data currently available on the cellular basis for chemotherapy response in these tumors by systematically following cisplatin—presumably the most active drug in the treatment of this disease—on its course from entering the cell to the execution of apoptosis. The emerging picture points towards a multifactorial explanation for the unique chemosensitivity of germ-cell tumors, including a lack of export pumps, an inability to detoxify cisplatin and repair the respective DNA damage, and an intact apoptotic cascade not disturbed by anti-apoptotic stimuli. Even though no uniform pattern of relevant resistance factors has been identified in patients suffering from refractory disease, a significant number of these cases may be caused by defects in the DNA mismatch repair pathway.

Key words: apoptosis, cell cycle control, chemotherapy resistance, cisplatin, germ-cell tumors

Introduction

Germ-cell tumors (GCTs) of adolescent and young males represent a histologically heterogeneous group of neoplasms derived from the germ-cell lineage. Most tumors occur within the gonads; ~5% of GCTs develop at extragonadal locations along the midline of the body [1, 2]. In western countries, testicular GCTs account for up to 60% of all malignancies diagnosed in male patients between 20 and 40 years of age [3]. Based on histological and clinical characteristics, these GCTs are divided into seminomas and nonseminomas. Seminomas are composed of uniform cells resembling primordial germ cells/gonocytes [4]. Nonseminomas contain one or more histological subtypes representing various differentiation lineages and stages of embryonic development. Embryonal carcinoma cells represent the stem cell component, which has the potential to differentiate towards extra-embryonic tissues (yolk sac tumor and choriocarcinoma) and embryonic tissues with mesenchymal, epithelial or neuronal components (immature and mature teratoma) [5].

Germ-cell tumors are highly sensitive to chemotherapy. Even in patients with metastatic disease, cure rates of 80% can be achieved with multiagent, cis-diamino-dichlorid-platin (cisplatin; CDDP)-based combination chemotherapy followed by secondary resection in the case of residual tumor lesions [6]. Only mature teratoma elements do not share the general chemosensitivity of GCTs, despite an identical genetic constitution. Due to intrinsic chemotherapy resistance, mature teratoma can be found in ~30–40% of residual lesions after chemotherapy for non-seminomatous GCTs [7]. Regardless of their benign behavior, complete resection of residual mature teratoma is mandatory to prevent transformation into secondary non-germ-cell malignancies.

Notwithstanding the overall good prognosis, 10–30% of patients diagnosed with metastatic GCT will not achieve a durable complete remission after initial treatment, either due to incomplete response or relapse. These patients become candidates for salvage treatment, often involving high-dose chemotherapy followed by autologous stem cell transplantation. Long-term disease-free survival rates of 30–50% have been reported with this approach [8]. Once a patient has also failed salvage therapy, further treatment is palliative in most cases [9].

Based on this clinical background, the understanding of the mechanisms of chemosensitivity and resistance of tumor cells is becoming more important in order to further improve therapeutic outcome. A more accurate prediction of treatment outcome may help to avoid under- or overtreatment. As the tools that influence specific cellular pathways are evolving, they may allow individual resistance mechanisms to be reversed or overcome, which might help to cure more patients in the future.

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This review summarizes the current knowledge on the biological basis of treatment response in GCTs. For reasons of clarity, the considerations focus primarily on the mode of action of CDDP and possible resistance mechanisms, as CDDP is the most important drug in the systemic treatment of this disease. Nevertheless, some of the observations will also apply for other cytotoxic substances. In order to understand the molecular basis of treatment sensitivity and resistance in invasive GCTs, this review follows the course of CDDP from cellular uptake to execution of apoptosis (Figure 1). Alternative cellular and extracellular mechanisms of resistance are subsequently discussed. Finally, a brief discussion of the possible explanations for the intrinsic resistance of mature teratomas is given.

Mechanisms affecting the intracellular CDDP concentration

The intracellular concentration of active CDDP can be lowered by a reduction in uptake, an increase in drug export or an increase in intracellular detoxification. Recent data suggest a role for copper transporter Ctrl in cellular CDDP uptake, which might be responsible for part of the CDDP entering into the cell. Cell lines lacking Ctrl show a reduced sensitivity towards CDDP [10]. However, most authors assume a passive or facilitated diffusion of CDDP into the cell [11]. In the latter case, reduced uptake could hardly play a role in drug resistance. Although CDDP is not a substrate for P-glycoprotein, the product of the multidrug resistance gene MDR, other members of the ATP-binding cassette superfamily of transporters (ABC transporters) do show affinity for CDDP, in particular when conjugated to glutathione. Thus, overexpression of these pumps can result in resistance to the drug, as has been demonstrated for different tumor cell lines [12]. Since these pumps can be inhibited pharmacologically, information about the precise cellular mechanism might offer ways to circumvent drug resistance in the future [13, 14], even though the experience with PSC833, an inhibitor of P-glycoprotein that has been extensively demonstrated in GCT cell lines has been attributed to the low intrinsic capacity of the respective DNA-repair pathway, i.e. NER [27]. However, in two sublines of invasive GCT-derived cell lines indicating a low capacity of the respective NER demonstrated in GCT cell lines has been attributed to low levels of xeroderma pigmentosum complementation group A protein (XPA) [29]. Alternatively, it has been proposed that the DNA adducts could be concealed by testis-specific high mobility group (HMG)-box proteins preventing damage detection and repair by NER factors [30]. The finding of a low NER capacity itself and the potential clinical relevance have not been confirmed in samples from patients with GCTs. It is conceivable that a low NER activity contributes to the overall chemosensitivity of GCTs. Whether this is—in clinical reality—determined by low levels of XPA or by alternative means remains to be proven. Even though theoretically possible, there are no data suggesting that changes in NER capacity play a critical role in tumors of patients failing chemotherapy.

DNA repair pathways

The second group of resistance factors to be discussed includes those which are involved in the repair of CDDP-induced DNA damage before a cell death program is activated. Of the various DNA repair mechanisms, the nucleotide excision repair pathway (NER) is supposed to be of major importance when CDDP exposure has resulted in covalent DNA lesions that distort the DNA helix [26].

CDDP is removed slowly from genomic DNA in different GCT-derived cell lines indicating a low capacity of the respective DNA-repair pathway, i.e. NER [27]. However, in two sublines with an acquired resistance to CDDP, DNA repair was unchanged compared with their parental lines [28]. The low intrinsic capacity of the NER demonstrated in GCT cell lines has been attributed to low levels of xeroderma pigmentosum complementation group A protein (XPA) [29]. Alternatively, it has been proposed that the DNA adducts could be concealed by testis-specific high mobility group (HMG)-box proteins preventing damage detection and repair by NER factors [30]. The finding of a low NER capacity itself and the potential clinical relevance have not been confirmed in samples from patients with GCTs. It is conceivable that a low NER activity contributes to the overall chemosensitivity of GCTs. Whether this is—in clinical reality—determined by low levels of XPA or by alternative means remains to be proven. Even though theoretically possible, there are no data suggesting that changes in NER capacity play a critical role in tumors of patients failing chemotherapy.

In contrast to the NER, the base excision repair pathway (BER) contributes to the elimination of small base alterations that do not distort the DNA helix. In the context of GCT treatment, this type of damage is inflicted by ionizing radiation and bleomycin [31].
Figure 1. Schematic representation of the mechanisms potentially influencing cisplatin activity. (A) Model of a germ-cell tumor cell showing sensitivity to cisplatin. The cellular composition favors cell death on cisplatin exposure by a lack of multiple potential resistance mechanisms. Continuous arrows indicate the path to cell death, dashed arrows indicate potential resistance mechanisms. (B) Model of a germ-cell tumor cell with potential means of chemotherapy resistance highlighted. In mature teratomas it is most likely that multiple factors contribute to the resistant phenotype. ABC, ATP binding cassette; DR, death receptor; GSH, glutathione; LRP, lung resistance protein.
The BER has been investigated in GCT-derived cell lines in correlation to their sensitivity to bleomycin. In vitro overexpression of Ape1/ref1 resulted in a two-fold resistance to bleomycin [32]. However, as the majority of investigated GCTs displayed strong immunohistochemical staining for this factor, a major effect of the mere protein level in determining resistance to bleomycin can be ruled out in view of the exquisite chemosensitivity of GCTs.

**Failure to initiate apoptotic cascades upon DNA damage recognition**

In addition to their repair capacity, factors of the various DNA-repair pathways are capable of initiating apoptotic pathways [33]. Therefore, the discrimination between this group of potential resistance mechanisms and the repair pathways is arbitrary. However, the effector role of specific pathways activated by platinum-DNA adducts in GCTs is probably primed in one way or other, thus justifying a separate discussion of both effects.

As far as CDDP-induced damage is concerned, the DNA mismatch repair pathway (MMR) seems to act as a link between damage recognition and initiation of apoptosis [34]. As defects in MMR factors lead to instability of short repetitive DNA sequences called microsatellites, the analysis of microsatellites provides information about the functional capacity of the MMR system. Losses or defects of MMR factors can confer resistance to CDDP, alkylating agents, methotrexate and the topoisomerase II inhibitor doxorubicin, as shown in various experimental systems [35, 36]. This resistance could either be explained by the acquisition of secondary mutations—for example, in effectors of apoptosis due to genetic instability—or alternatively, assuming a critical role of the MMR in linking the detection of damage to apoptosis, respective defects would directly result in failure to initiate the apoptotic cascade. Germ-cell tumors have been found to be microsatellite stable in previous studies [37, 38]. These findings were confirmed in a series of 100 unselected GCTs, where only 6% of cases showed MSI in at most one out of eight investigated loci. In contrast, five of 11 (45%) specimens from patients with refractory disease had unstable microsatellites, four of them in at least two loci, suggesting that failure to initiate apoptosis due to defects in MMR might contribute to resistance [39]. The number of refractory cases investigated should be expanded to confirm the clinical relevance and the predictive value of these findings.

A dual role in stress response is also known for P53. P53 mediates a G₁/S-phase cell cycle arrest via transactivation of P21, allowing time for DNA repair, which can involve GADD45 as another P53 target. In addition, P53 can lead to apoptosis via the mitochondrial pathway, e.g. by induction of Bax [40]. In contrast to other solid tumors, mutations in p53 are hardly ever found in GCTs. At the same time, P53 is detected immunohistochemically in most GCTs. Therefore, a high level of wild-type P53 in GCTs has commonly been regarded as the biological explanation for their chemosensitivity. The experimental evidence supporting this idea is partly based on studies in the mouse teratocarcinoma cell line P19 [41, 42], while data on human cell lines are conflicting [43, 44].

Two studies have analyzed the p53 status in tumor samples from refractory patients. In a study on relapsed GCTs, p53 mutations were detected in four of 28 tumors, three of them were mature teratomas, the remaining one a secondary non-germ-cell malignancy derived from teratoma [45]. All mutation-containing tumors thus belonged to intrinsically chemotherapy-resistant histological subgroups. Therefore, the contribution of p53 status to clinical behavior is difficult to estimate. In a second study, no p53 mutations were found in a group of 18 refractory cases, their P53 levels were comparable with those of sensitive and unselected cases. The difference between these studies might be explained by the different histologies investigated, with less samples of mature teratoma and no secondary non-germ-cell malignancies in the latter study. In conclusion, the level of P53 alone cannot explain the chemotherapeutic sensitivity of GCTs. For the majority of refractory GCT patients, p53 mutations are also unlikely to be the cause of resistance.

**Execution of apoptosis**

Finally, resistance can emerge from failure to execute apoptosis despite initiation of the apoptotic cascade, either due to predominance of anti-apoptotic factors, such as certain members of the Bcl-2 family, or defects in downstream effectors of apoptosis e.g. caspases. For example, the level of Bcl-2 has been correlated with poor chemotherapy response in patients with myeloid leukemia and small-cell lung cancer [46].

In GCT-derived cell lines the sensitivity towards etoposide—after CDDP, the second most commonly used drug for GCTs—was ascribed to a high ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2, both members of the Bcl-2 family acting downstream of P53 [47]. No such correlation was found in four different GCT cell lines treated with CDDP. After exposure to the drug, no induction of Bcl-2 and Bax was observed [48]. Two studies on GCT samples have confirmed the high Bax:Bcl-2 ratio in invasive components. However, no correlation of Bax or Bcl-2 with clinical outcome was evident in either study [18, 49].

In our own investigation, the Bcl-2 signal was strong in mature teratomas, whereas Bax staining was hardly seen. Investigating the relation between the apoptotic index and P53 or P21 in responding and refractory GCTs, no correlation of any of the parameters and clinical outcome was observed. P53 and the apoptotic index were correlated; P21 was hardly detected [18]. These findings argue against anti-apoptotic influences downstream in a P53-dependent apoptotic pathway—at least not the one used for spontaneous apoptosis—as the common mechanisms of resistance. The small heat shock protein HSP27 has been shown to inhibit caspase 9 activation upon cytochrome c release. In this context, the low levels of HSP27 found in GCTs may contribute to chemosensitivity at the level of execution of apoptosis [50].

**Regulatory proteins**

An increase in the understanding of the biology of tumor cells has lead to the identification of various oncogenes, such as c-myc, c-fos, H-ras and c-jun, that can modify the growth characteristics
and stress responses of tumor cells [11]. The ras proto-oncogene is involved in the regulation of proliferation, apoptosis and survival. The impact of mutated Ras on chemotherapy-resistance has been discussed controversially. K-ras maps to the short arm of chromosome 12 that is usually overrepresented in GCTs. However, ras mutations are rare in GCTs, and if present, they do not result in poor treatment outcome [51]. Downstream in the Ras/MAP-kinase pathway, c-Fos is a component of the activator protein 1 (AP-1) transcription factor. Overexpression of c-Fos resulted in resistance to CDDP in different tumor models [52]; transfection with antisense or a dominant-negative construct resulted in sensitization of resistant cells to CDDP [53]. However, data on c-Fos/AP-1 in cell lines or tumor samples of GCTs are not currently available.

**Aneuploidy and chemotherapy response in GCTs**

Whereas the putative mechanisms of chemotherapy resistance described so far are based on mutations or changes in the expression of specific genes, an alternative means of developing chemotherapy resistance has been proposed. According to this model, aneuploidy—the most consistent genomic alteration in solid tumors—allows the tumor cells to achieve a resistant phenotype by chromosome reassociation [54, 55]. Various studies on different tumor entities support at least a correlation between aneuploidy and prognosis, even though this correlation does not necessarily depend on differences in chemotherapy response. The correlation between aneuploidy and treatment outcome has not yet been investigated in GCTs. Differences in the degree of aneuploidy between responding and non-responding cases have been reported only in case reports. Nevertheless, the biological diversity of GCTs allows for some considerations in this regard. Nearly all seminomas and all nonseminomas of the adult are aneuploid [56, 57]. Whereas most of the invasive components will respond favorably to chemotherapy, mature teratoma is intrinsically chemotherapy resistant. In contrast, mature teratomas in the infant are known to be diploid, and chemotherapy resistant. On progression to invasive yolk sac tumors, a certain degree of aneuploidy occurs. In contrast to the diploid mature teratomas of the infant, the yolk sac tumors can be cured by chemotherapy, as is the case in seminomas and nonseminomas in the adult. These data seem to exclude a clear-cut correlation between aneuploidy and chemotherapy response in GCTs.

Interestingly, as an exception to the aneuploidy of GCTs in the adult, van Echten et al. [58] described pseudodiploid GCTs of the retroperitoneum and the mediastinum with the histology of immature teratoma as an entity with a highly chemotherapy resistant behavior. As it is known from different tumor entities that microsatellite instability and aneuploidy are virtually exclusive, it is tempting to speculate that the postulated refractory entity of extragonadal GCTs might derive their chemotherapy resistance from microsatellite instability.

**Telomeres**

Telomeres are repetitive DNA sequences protecting chromosome ends from fusion during cell division. Their length decreases with each cell division in somatic cells. The loss of the protective telomeres results in senescence. In order to ensure a sufficient telomere length for subsequent generations, germ cells possess a high endogenous activity of telomerase—an enzyme able to maintain telomere length. High telomerase activity has also been found in GCTs [59], and an inverse correlation has been described between telomerase activity and differentiation in cell lines derived from GCT.

Recently, the action of CDDP has been correlated with telomere length and telomerase activity in different models. Kiyoyuka et al. [60] found a correlation between expression of human telomerase reverse transcriptase—a component of telomerase complex—and sensitivity to CDDP in ovarian cancer cell lines. In HeLa cells, a reduction in telomere length was found following treatment with CDDP [61]. In cell lines derived from GCTs, Burger et al. [62] reported an inhibition of telomerase activity by CDDP. However, the decrease of telomerase activity in the respective cell lines did not correlate with CDDP sensitivity [63]. As telomerase inhibitors are currently being developed, a thorough understanding of the telomere biology will help to identify targets for therapeutic intervention. The combination of CDDP with telomerase inhibitors might offer a potential approach for treating refractory GCTs.

**Extracellular influences on the efficacy of chemotherapy**

Thus far, only cellular factors affecting platinum activity have been reviewed, yet it is obvious that a drug first has to reach the tumor cell in order to exert its action. An increase in the diffusion distance from vessels to tumor cells by changes in the extracellular matrix may contribute to a reduction in intracellular CDDP concentration. Thus, extracellular factors, such as microvessel density or the composition of the extracellular matrix, will additionally determine the efficacy of treatment. Other changes in the microenvironment can directly affect cellular functions. For example, hypoxia results in HIF1-mediated induction of MDR1 in T84 and Caco-2B cells [64], and induction of P53 with a consecutive P21-mediated cell cycle arrest [65]. Large metastases in patients with GCTs will at least be partly hypoxic, as indicated by extensive necrotic areas in many of these tumors. However, no systematic investigations of extracellular influences on chemotherapy efficacy in GCTs are available yet.

**Intrinsic chemotherapy resistance in mature teratoma**

Mature teratomas are clinically resistant to the effects of chemotherapy. However, no genetic differences between mature teratoma and the invasive components of GCTs have been demonstrated so far. Thus, it is likely that their resistant phenotype is induced by changes in the expression level of different genes in the course of somatic differentiation. In contrast to invasive GCT
components, epithelial tissues of mature teratomas were mostly positive for MDR, MRP2, BCRP and LRP by immunohistochemistry. Furthermore, the presence of GSTp has been demonstrated in tissue of mature teratomas [18].

A further difference can be observed in the expression of the cell cycle-associated proteins P21 and RB. In contrast to the invasive components of GCTs, mature teratomas have been found to express RB and P21, suggesting an ability for G1/S cell cycle arrest [18, 66]. No correlation between the number of P53-positive cells and the apoptotic index was found in a small series of mature teratomas [18]. In addition, unlike invasive tumors, mature teratomas show a very low Bax:Bcl-2 ratio. Together, these findings suggest that mature teratoma cells may respond to CDDP-induced DNA damage by P21 cell cycle arrest rather than by Bax-mediated apoptosis.

So far, the available data indicate that the resistant phenotype of mature teratomas is a result of the concerted expression of different resistance factors affecting cellular functions, such as drug export, cell cycle control and regulation of apoptosis. Most likely, the pattern is a consequence of somatic differentiation and will not be restricted to the factors mentioned above.

Conclusions and future perspectives
Due to their diverse histology, the relation to embryonic development and the differences seen in response to chemotherapy, with high cure rates in most patients but resistance in a small percentage of cases, GCTs provide a fascinating model for experimental and clinical research in the field of oncology. Despite an increasing body of information on the different aspects of chemotherapy resistance, the whole picture is just beginning to evolve. The exquisite chemosensitivity of GCTs seems to be the consequence of a favorable spectrum of factors, including a lack of drug export and detoxification mechanisms, low DNA-repair capacity, sensitive DNA-damage detection systems, with subsequent initiation and execution of apoptotic pathways in the vast majority of cases.

In contrast to the invasive tumor components, in multiple regards, multiple factors seem to contribute to the intrinsic chemotherapeutic resistance in mature teratomas. The phenotype of mature teratomas is most likely a consequence of somatic differentiation rather than a tumor-specific alteration. It is conceivable that the development of a systemic treatment approach that is able to eliminate mature teratomas without, at the same time, affecting regular somatic tissues of identical histological differentiation, will be difficult. Any such treatment would elicit unacceptable toxicities. Complete surgical resection of residual teratoma after chemotherapy seems to remain an adequate approach.

For the development of a resistant phenotype in invasive GCT components, no uniform explanation can be offered. A significant proportion of these cases may be caused by defects in the MMR system resulting in microsatellite instability. Whether the resistance is the direct consequence of failure to detect CDDP-induced DNA damage and to initiate the apoptotic cascade or an indirect consequence of the accumulation of mutations affecting apoptotic effectors remains to be determined. Overexpression of export pumps or enzymes detoxifying drugs might confer resistance only in some cases, but does not account for the majority of patients suffering from chemotherapy-resistant GCT.

In view of the variety and complex interactions of pathways resulting in or preventing cell death, methods with the ability to investigate multiple factors at a time are probably necessary to predict the response of individual tumors to chemotherapeutic regimens.

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