Failure of Activation of Caspase-9 Induces a Higher Threshold for Apoptosis and Cisplatin Resistance in Testicular Cancer


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ABSTRACT

Testicular germ cell cancer is one of the very few cancers that are highly sensitive to and curable by cisplatin-based chemotherapy even in an advanced stage. However, in a few cases resistance to cisplatin occurs and patients subsequently die from progressive disease. The molecular basis for this resistance remains to be determined. Using two cisplatin-sensitive (2102EP and H12.1) and one cisplatin-resistant human testicular germ cell cancer cell line (1411HP), we investigated molecular mechanisms in the induction of apoptosis after cisplatin-treatment focusing on the cleavage and activation of caspase-2, caspase-3, caspase-7, caspase-8, and caspase-9.

The cell line 1411HP showed a 3.3-fold cisplatin resistance when compared with the sensitive cell lines 2102EP and H12.1 by IC50, which was treatment schedule independent (2- or 24-h incubation). Cisplatin resistance was associated with substantially decreased apoptosis in vitro and in derived nude mice xenografts as determined by Apo 2.7 detection, DNA-laddering, immunohistochemistry of active caspase-3, and terminal deoxynucleotidyl transferase-mediated nick end labeling assay. Total DNA platination as assessed by ELISA after cisplatin treatment in equimolar doses did not differ between cisplatin-resistant or -sensitive cells. In separate analysis of cells of early and late apoptotic stages, initiation of cisplatin-induced apoptosis appeared to be rather mediated by caspase-9 than by caspase-8. Resistant 1411HP cells failed to activate caspase-9 during the induction of apoptosis after cisplatin treatment at the IC50 dose. Interestingly, inhibition of caspase-9 in sensitive H12.1 almost completely blocked apoptosis and induced cisplatin resistance to the same extent as in 1411HP so that apoptosis could only be induced by 3.3-fold higher cisplatin doses. Furthermore, in caspase-9 blocked cells, initiation of apoptosis occurred in a caspase-9-independent manner accompanied by activation of caspase-2 and caspase-3, which are intrinsic characteristics of resistant 1411HP cells. Failure of caspase-9 activation and cisplatin resistance was independent of the expression of p53, Bel-2 family proteins, Fas receptor, and Fas ligand. In conclusion, failure of activation of the caspase-9 pathway induces a higher cellular threshold for cisplatin-mediated induction of apoptosis in testicular cancer cells. However, this higher threshold can be overcome by higher cisplatin doses, conceivably by using an alternative, cisplatin-independent apoptotic pathway. This supports the current clinical strategy of high-dose chemotherapy in patients with chemoresistant germ cell tumors. However, additional defining and eventually targeting the exact molecular mechanism blocking caspase-9 activation might lead to more selective therapeutic approaches to overcome cisplatin resistance in germ cell cancer.

INTRODUCTION

Testicular germ cell tumors, which are the most common malignancies in young men, represent one of the very few cancer types that are highly chemosensitive and therefore highly curable by chemotherapy even in an advanced stage (1, 2). The anticancer agent cisplatin plays a pivotal role in the treatment of testicular cancers. Currently, the cure rate of metastatic testicular cancer exceeds 80% and in certain low-risk stages even 90%. However, in a few cases, cisplatin-based chemotherapy fails to induce tumor regression and patients eventually die from progressive tumor growth. The reasons for resistance in these few cases remain obscure, particularly with regard to the exceptional chemotherapeutic sensitivity of this cancer type in general.

Cisplatin was shown to induce programmed cell death in vitro (3). Substantial evidence indicates a general association between susceptibility to apoptosis and response to chemotherapy (4, 5). It has been suggested that drug-target interactions are only the first step in the commitment to programmed cell death and that chemosensitivity is not because of cellular damage per se but to differences in the response of cells to damage (6). Different cell types vary profoundly in their susceptibility to the induction of apoptosis and understanding what determines cellular thresholds for apoptosis could be important for cancer therapy (5). Testicular cancer cells are prone to and may already be primed to undergo apoptosis (7). Obviously, testicular cancer cells usually have a low apoptosis threshold and thus a high inclination toward therapy-induced apoptosis. It is of particular interest to further elucidate the molecular mechanisms that account for the high susceptibility to undergo apoptosis in this cancer cell type as well as those involved in the occurrence of resistance in some few cases. As yet, analysis of various pharmacological and molecular parameters have failed to fully explain this phenomenon.

Apoptosis, the evolutionarily conserved form of active cell death, requires specialized machinery. A central component of the apoptotic machinery is a proteolytic system that involves a family of proteases named caspases (8). These enzymes participate in a cascade that is triggered in response to proapoptotic signals and culminates in the cleavage of a downstream set of proteins, resulting in overall disassembly of the cell (9, 10). From a functional standpoint and with regards to their predecessors, these caspases can be divided into two groups: large prodomain containing upstream initiators (caspase-2, caspase-8, caspase-9, caspase-10), which may initiate the proteolytic cascade, and small prodomain containing downstream effectors (caspase-3, caspase-6, caspase-7), which in turn can amplify the signal by cleaving initiator-caspases and kill the cell by cleaving key intracellular targets (9, 10). To date, two major caspase pathways, headed by caspase-8 and caspase-9, have been described that mediate distinct sets of signals (11, 12). Caspase-8 is involved in death receptor-mediated apoptosis, such as those involved in the occurrence of resistance in some few cases. The pathway initiated by caspase-9 is thought to mediate chemically induced apoptosis. This cascade is triggered by the release of cytochrome c from mitochondria and leads to the formation of the apoptosome that activates caspase-9, which subsequently activates effector caspases (15, 16). However, several studies also indicate an important role for the CD95/Fas-system in drug-induced apoptosis (17), which implies that caspase-8 is another caspase critical in the chemically induced apoptotic pathway. Furthermore, a recent report provided evidence for the existence of a novel drug-inducible apoptotic pathway in which activation of caspase-8 forms the apical step, thus challenging the assumption that drug-induced apoptosis is pref-
Differentially mediated in caspase-9 (18). However, this pathway was shown to be independent of death receptors, but similar to the caspase-9 pathway, it has also been shown to be mitochondria dependent.

The proteins of the Bcl-2-family play a crucial role in the function of mitochondria during the apoptotic process and may act as balancing factors for the apoptotic program (19, 20). An additional important mediator of apoptosis is the tumor suppressor protein p53. Functional inactivation of p53 in human tumors by mutations or interactions with cellular or viral proteins has been associated with resistance to genotoxic agents commonly used in anticancer therapies (21). Consistently, sensitivity of human testicular tumors to drug-induced apoptosis has been associated with the existence of functional p53 and a high Bax:Bcl-2 ratio (22).

In this study, we investigated differential responses to cisplatin in sensitive and resistant testicular cancer cells, particularly focusing on the activation of caspases. Here, we demonstrate that in testicular cancer cells cisplatin-induced apoptosis is preferentially mediated by caspase-9 and not by caspase-8 and that resistant testicular cancer cells fail to activate caspase-9 during the induction of apoptosis. As simulated in sensitive testicular cancer cells, such a defect does not ultimately block apoptosis but rather induces a higher cellular threshold for apoptosis induction such that a higher cisplatin dose is required to trigger apoptosis. Additional analysis demonstrated that differential sensitivity to cisplatin and differential activation of caspase-9 was independent of the expression of key regulators of apoptosis such as p53, Bcl-2-family proteins and the Fas-system.

MATERIALS AND METHODS

Cell Lines. Human testicular cancer cell lines 2102EP (23) and 1411HP (24) were kindly provided by Dr. Peter W. Andrews (University of Sheffield). The human testicular cancer cell line H12.1 was established from orchietomy specimen of a 19-year-old previously untreated patient (25). Cell lines were maintained as monolayer cultures in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (Biochrom KG Seromed, Berlin, Germany) and streptomycin/penicillin (Life Technolgies, Inc.). Cultures were grown at 37°C in a humidified atmosphere of 5% CO₂/95% air.

Cytotoxicity Assays and Schedule-specific Determination of IC₅₀

Dose-response curves of the cell lines to 0.01–100 µm cisplatin (Sigma) were established using the SRB1 microculture colorimetric assay as described by Skehan et al. (26). Briefly, cells were seeded into 96-well plates on day 0 at cell densities previously determined to ensure exponential cell growth during the period of the experiment. On day 1, cells were treated with the appropriate cisplatin concentrations for 24 and 24 h and then washed and supplemented with drug-free medium. The percentage of surviving cells relative to untreated controls was determined on day 5. The cisplatin concentration that inhibited drug-free medium. The percentage of surviving cells relative to untreated the period of the experiment. On day 1, cells were treated with the appropriate cell densities previously determined to ensure exponential cell growth during the induction of apoptosis. As simulated in sensitive testicular cancer cells, such a defect does not ultimately block apoptosis but rather induces a higher cellular threshold for apoptosis induction such that a higher cisplatin dose is required to trigger apoptosis. Additional analysis demonstrated that differential sensitivity to cisplatin and differential activation of caspase-9 was independent of the expression of key regulators of apoptosis such as p53, Bcl-2-family proteins and the Fas-system.

DNA Fragmentation. Adherent and floating cells were separately analyzed. Cells were washed twice with PBS and lysed in lysis-buffer [100 mm Tris-HCl (pH 8.0), 20 mm EDTA, 0.8% SDS]. After treatment with RNase A for 2 h, and proteinase K (Roche Molecular Biochemicals) overnight, lysates were mixed with DNA loading buffer. Probes were run on a 1.5% agarose gel followed by ethidium bromide staining and rinsing with distilled water. DNA ladders were visualized under UV light and documented by photography.

DNA Platination. Floating cells were discarded and adherent cells were harvested, washed twice with PBS, and their DNA was extracted using the QIAamp Blood Kit (Qiagen). DNA was denatured and the overall platination was measured by a competitive ELISA previously described by Tilby et al. (28, 29). The monoclonal antibody CP 9/19 as well as the coating solution and DNA-standard were kindly provided by Dr. Michael J. Tilby (Newcastle, United Kingdom).

Western Blot Analysis. Adherent and floating cells were analyzed separately. Cells were rinsed twice with PBS and lysed in RIPA buffer [50 mm Tris-HCl (pH 8.0), 150 mm NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS] supplemented with a protease inhibitor mixture (Sigma). Insoluble components were removed by centrifugation and protein concentrations were measured (Bio-Rad protein assay; Bio-Rad). After boiling for 5 min in SDS loading buffer [62.5 mm Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 100 mm DTDT], 60 µg protein/lane was separated by SDS-PAGE and electroblotted onto nitrocellulose membrane (Bio-Rad). Equal protein loading was controlled by Ponceau S staining (Sigma). Membranes were blocked with 5% nonfat dry milk in PBS + 0.1% Tween 20 for 1 h and probed for 2 h with the following antibodies diluted in PBS + 0.1% Tween 20/5% milk: Fas (C-20) rabbit polyclonal IgG (1 µg/ml); Fasl-N (N-20) rabbit polyclonal IgG (1 µg/ml); p53 (Do-1) mouse monoclonal IgG (0.1 µg/ml); Bak (G-23) rabbit polyclonal IgG (1 µg/ml); Bcl X (S-18) rabbit polyclonal IgG (1 µg/ml; Santa Cruz Biotechnology); Bcl 2 (Clon 124) mouse monoclonal IgG (2 µg/ml); Bax rabbit polyclonal IgG (1 µg/ml; Dako); caspase-3 rabbit polyclonal IgG (1:2000); caspase-7 (Ab-1) rabbit polyclonal IgG (1 µg/ml); caspase-9 (Ab-2) mouse monoclonal IgG (1 µg/ml; Oncogene Research Products); caspase-2 (clon 35) mouse monoclonal IgG (1 µg/ml; Transduction Laboratories); and caspase-8 (12F5) mouse monoclonal IgG (1 µg/ml; BioSource). Immunocomplexes were visualized by enhanced chemiluminescence (Amer sham Pharmacia Biotech) using horseradish peroxidase-conjugated antioimmunoglobulin or antirabbit IgG (Santa Cruz Biotechnology).

Immunohistochemistry. Nude mice bearing the cisplatin sensitive H12.1-tumors or cisplatin-resistant 1411HP tumors received daily i.p. injections of 2 mg/kg/day cisplatin from days 1 to 5. On day 6, after onset of treatment when H12.1 tumors were always responding, both tumors and untreated controls were removed, fixed in Bouin-solution, and embedded in paraffin. Sections of 5 µm were deparaffinized and rehydrated. The slides were treated with 3% hydrogen peroxide in methylalcohol to quench endogenous peroxidase activity and incubated with 3% BSA in PBS to decrease nonspecific binding of antibodies. Subsequently, slides were incubated with rabbit polyclonal anti-caspase-3 antibody (PharMingen) overnight at 4°C. After washing three times with PBS, slides were treated with peroxidase-labeled antirabbit IgG (Santa Cruz Biotechnology) followed by washing. 3,3'-Diaminobenzidine (Dako) was used as the substrate for visualization of the immunocomplex. Slides were counterstained with hematoxylin and mounted with Depex (Dako).

Transfection. 1411HP cells were seeded in 6-well plates 24 h before transfection and semiconfluent monolayers were transfected with the expression vector pCR3.1, containing or lacking the human FAS gene, using Doper transfection reagent (Roche Molecular Biomicals). The vector constructs were kindly provided by Dr. Kentaro Yamada (Kurume University; Ref. 30). Cells were incubated in nonselective growth medium containing the DNA/Dosper mixture for 6 h followed by rinsing. After 24-h incubation in normal growth medium, cells were treated with G418 (Roche Molecular Biochemicals) for 24 h, and proteinase K (Roche Molecular Biochemicals) overnight, lysates were mixed with DNA loading buffer. Probes were run on a 1.5% agarose gel followed by ethidium bromide staining and rinsing with distilled water. DNA ladders were visualized under UV light and documented by photography.

Caspase Activation Assays and Inhibition of Caspase-9. Adherent and floating cells were analyzed separately. Cells were washed twice with PBS, lysed in cell lysis buffer (BioSource), and incubated for 10 min on ice. All subsequent steps were performed on ice. After centrifugation, cell extracts were transferred to fresh tubes, and protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad). Each 50-µl cell extract containing 100 µg of protein were combined with equal volumes of 2× reaction buffer

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1 The abbreviations used are: SRB, sulforhodamine-B; FasR, Fas receptor; Fasl, Fas ligand.
RESULTS

Differential Sensitivity to Cisplatin and Mode of Cell Death Is Independent of Treatment Schedule. IC_{50}s of 2- and 24-h treatment schedules were determined for each testicular cancer cell line by semilogarithmic dose-response plots. In terms of IC_{50}s, cell line 1411HP showed a 3.3-fold resistance to cisplatin when compared with the sensitive cell lines 2102EP and H12.1, independent of the treatment schedule used (Table 1). For IC_{50}s, the resistance approached 10-fold (data not shown).

Determination of apoptotic cell death was performed by DNA gel electrophoresis and by flow cytometry using the apoptosis marker Apo2.7. Independent of the treatment schedule, all three cell lines underwent apoptotic cell death after treatment with the appropriate IC_{50} doses of cisplatin as demonstrated by the occurrence of typical DNA fragmentation and detection of Apo 2.7 in floating cells (Fig. 1). Furthermore, apoptotic cell death of the floating cells was confirmed by a trypan-blue exclusion test (data not shown) because as cells dying by apoptosis are still able to exclude dye for a distinct time.

Resistance to Cisplatin Is Associated with Decreased Induction of Apoptosis. In all three cell lines, the apoptotic hallmark of DNA fragmentation was associated with the presence of Apo2.7 antigen, which was solely found in the floating cell population (Fig. 1). Thus, the measurement of Apo2.7 expression level was a useful tool to quantify an apoptotic cell population in our testicular cancer cell lines. As summarized in Table 2, a substantial apoptotic cell population was observed in the sensitive cell lines 2102EP and H12.1 after treatment with 30 \( \mu \)M cisplatin for 2 h. However, even at 48 h, no apoptotic cell population was apparent in the resistant cell line 1411HP in which almost nonfloating cells could be found after 5 days when almost all cells of the sensitive cell lines were apoptotic (data not shown).

However, exposing 1411HP cells to 100 \( \mu \)M cisplatin for 2 h induced apoptosis with the same characteristics as in the other sensitive cell lines. Thus, in the cisplatin resistant cell line 1411HP, 3.3-fold higher molar doses of cisplatin were required to initiate apoptosis and to achieve the same cytotoxic effect as lower doses in the sensitive cell lines (equitoxic doses). To mimic clinically relevant treatment conditions regarding cisplatin concentrations in a manner analogous to the most commonly used chemotherapy protocol in testicular cancer (31), we used the 24-h treatment schedule for most of the subsequent experiments as indicated.

Table 1 IC_{50} of testicular cancer cell lines treated with cisplatin (\( \mu \)M)

<table>
<thead>
<tr>
<th>Schedule</th>
<th>2102EP</th>
<th>H12.1</th>
<th>1411HP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDDP for 2 h</td>
<td>30</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>CDDP for 24 h</td>
<td>3</td>
<td>3</td>
<td>10</td>
</tr>
</tbody>
</table>

IC_{50}s were determined by analyzing semilogarithmic dose-response plots of SRB assays. Cell lines were treated with the cisplatin for 2 and 24 h, and the percentage of cell survival relative to untreated control was determined after 5 days. Values represent the mean of at least six independent experiments with a SD \( \leq 10\% \).

Table 2 Quantification of apoptotic cell population (percentage of all cells) by flow cytometry using the apoptosis marker Apo 2.7

<table>
<thead>
<tr>
<th>Treated/Untreated samples</th>
<th>30 ( \mu )M Cisplatin</th>
<th>100 ( \mu )M Cisplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>24 h after CDDP</td>
<td>17</td>
<td>27</td>
</tr>
<tr>
<td>48 h after CDDP</td>
<td>44</td>
<td>42</td>
</tr>
</tbody>
</table>

Apo 2.7 was measured by flow cytometry. Cell lines were treated with 30 \( \mu \)M cisplatin for 2 h, washed, and both adherent and floating cells were pooled and processed after 24 h and 48-h incubation in drug-free medium. Additionally, 1411HP cells were subjected to the same treatment schedule using 100 \( \mu \)M cisplatin. Apoptotic populations were calculated in percentage of the whole cell population. Values represent the mean of three independent experiments with a SD \( \leq 10\% \).

Cisplatin Resistance Is Not Associated with Decreased DNA Platination. To exclude that a mechanism limiting cisplatin-induced DNA damage was responsible for the resistance in 1411HP, we measured the overall DNA platination of the three cell lines at distinct time points using a competitive ELISA. As shown in Fig. 2, neither decreased DNA cisplatin adduct formation nor increased adduct removal occurred in the resistant cell line 1411HP after exposure to 30 \( \mu \)M cisplatin for 2 h. These findings demonstrated that the same severity of DNA damage readily triggered apoptosis in sensitive cells but was well tolerated by the resistant cell line. Treatment of the resistant 1411HP with equitoxic doses of 100 \( \mu \)M cisplatin for 2 h, led to a \(~ 10\)-fold increase in DNA platination when compared with 30 \( \mu \)M cisplatin (Fig. 2). This high cisplatin dose was required to induce the appearance of apoptotic cells after 24 and 48 h as seen in the sensitive cell lines after treatment with 30 \( \mu \)M cisplatin (Table 2). Thus, the resistant 1411HP cells have a higher threshold to initiate apoptosis than sensitive cells, and this threshold can only be overcome by a much larger amount of genotoxic DNA cisplatin adducts.
were abundant in the H12.1-tumor tissue (Fig. 3). Apoptotic cells detected after treatment with cisplatin in equimolar tumors and resistant 1411HP tumors differed greatly in the number of end labeling assay (Roche Molecular Biochemicals). Sensitive H12.1 tumors committed to undergo apoptosis and, therefore, represent a cell fraction in a very early stage in the apoptotic process. In contrast, floating cells represent the cell fraction in a late apoptotic stage and show all typical features of apoptosis (Fig. 1). Even if the floating cells are at a late, irreversible stage (DNA is fragmented) and, therefore, less relevant to the initial triggering events of apoptosis, the protein lysates of this fraction were analyzed as positive controls for apoptosis. In all three cell lines, each caspase tested was readily expressed and cleaved during the course of apoptosis as visualized by the decrease in procaspases and/or the occurrence of cleavage fragments in the floating cell fraction (Fig. 4). In addition, cleaved/activated forms of caspase-2 and caspase-3 were already detected in adherent cells, whereas activation of caspase-7 and caspase-8 was only observed in floating cells. Thus, caspase-8 appeared to play no critical role in initiating cisplatin-induced apoptosis in our testicular cancer cell model. Interestingly, the p35 fragment of active caspase-9 also occurred in adherent cells but only in the sensitive cell lines 2102EP and H12.1, whereas it could only be found in floating cells of the resistant cell line 1411HP. To confirm caspase activation in sensitive and resistant cells of early apoptotic stages, we measured the enzymatic activities of caspase-2, caspase-3, and caspase-9 in adherent cells by substrate cleavage assays using peptide substrates preferentially cleaved by these caspases (33, 34). As shown in Fig. 5, activity for caspase-2 and caspase-3 was observed in both, sensitive H12.1 and resistant 1411HP cells, whereas relevant caspase-9 activity was only observed in sensitive H12.1 cells. This correlates with the results of

Clinically Relevant Cisplatin Doses Do Not Induce Apoptosis and Tumor Regression in Resistant Cell Line. Determination of cancer cell sensitivity and resistance in cell lines is often considered to be of limited clinical relevance. We had previously shown that the sensitive cell line H12.1 and the resistant line 1411HP maintain their in vitro chemosensitivity characteristics toward cisplatin in vivo as nude mice tumors, under clinically relevant treatment conditions (32). Using this in vivo model, cisplatin treatment over 5 days could induce complete remissions in H12.1 tumors, whereas only growth retardations were observed in 1411HP tumors. On day 6 after onset of treatment, when all H12.1 tumors were already regressing, the number of apoptotic cells in H12.1 tumors and 1411HP tumors was determined by immunohistochemistry using an antibody which solely recognizes active caspase-3 (PharMingen). The results were confirmed by the terminal deoxynucleotidyl transferase-mediated nick end labeling assay (Roche Molecular Biochemicals). Sensitive H12.1 tumors and resistant 1411HP tumors differed greatly in the number of apoptotic cells detected after treatment with cisplatin in equimolar concentrations. As shown in Fig. 3, cells positive for active caspase-3 were abundant in the H12.1-tumor tissue (Fig. 3A) in contrast to the 1411HP tumors (Fig. 3B), where the number of apoptotic cells did not differ from untreated controls. Thus, it is conceivable that the induction of apoptosis by cisplatin was the main reason for tumor regression in our testicular cancer in vivo model. Furthermore, it demonstrated that clinically relevant cisplatin concentrations failed to overcome the higher threshold for apoptosis in 1411HP cells and thus could not induce apoptotic cell death and tumor regression.

Failure of Activation of Caspase-9 during Induction of Apoptosis in Resistant Cancer Cells. A key component of the apoptotic machinery is the proteolytic system of caspases. Because 1411HP cells contained a decreased susceptibility to undergo apoptosis leading to cisplatin resistance, we investigated the expression and cleavage (activation) of caspase-2, caspase-3, caspase-7, caspase-8 and caspase-9 in sensitive and resistant testicular cancer cells after treatment with appropriate (equitoxic) IC90 doses of cisplatin for 24 h. Western blot analysis was performed using separate lysates made from adherent cells and floating cells. Almost all cells treated with cisplatin at IC90 doses eventually undergo apoptosis. However, after treatment, cells remain adherent for some time until they detach from the tissue culture plate and start to float. Although adherent cells do not show phenotypic characteristics of apoptosis, they are already committed to undergo apoptosis and, therefore, represent a cell fraction...
the Western blot analysis and suggests that activation of caspase-9 is impaired in resistant testicular cancer cells during the induction of apoptosis. In floating cells of resistant 1411HP, caspase-9 was cleaved (Fig. 4) and likewise enzymatic activity of caspase-9 was observed (Fig. 5). Thus, cleavage and activation of caspase-9 occurred as a subsequent, more downstream event but played no critical role in triggering the apoptotic cascade in the resistant cells. However, the activity of caspase-9 in floating cells of resistant 1411HP was decreased when compared with the floating cells of sensitive H12.1 (Fig. 5). In addition, only a small increase of caspase-2 and caspase-3 activity was observed in the floating cells of 1411HP as compared with the adherent cells, whereas in the floating cells of H12.1, the activities of caspase-2 and caspase-3 were increased 2-fold (data not shown). This suggests that the impairment of caspase-9 activation in 1411HP cells effects the entire caspase cascade, presumably because of a lack of amplification loops. However, this did not prevent apoptosis in 1411HP after treatment with the appropriate (equitoxic) IC90 doses of cisplatin.

It is conceivable that the failure to activate caspase-9 caused the decreased susceptibility of 1411HP cells to initiate apoptosis in response to cisplatin-induced DNA damage that can readily trigger apoptosis in sensitive cells. Consequently, the apoptotic cascade in 1411HP cells has to be started by a variant, caspase-9-independent pathway, which conceivably requires more DNA damage for its initiation.

Blocking of Caspase-9 in Sensitive Cancer Cells Induces a Higher Activation Threshold for Apoptosis Leading to Cisplatin Resistance. To prove the role of caspase-9 as upstream caspase in our sensitive cell line H12.1, we inhibited caspase-9 using a peptide-based inhibitor. The inhibition of caspase-9 almost completely blocked the presence of apoptotic floating cells after treatment with 3 μM cisplatin for 24 h (IC90 of sensitive cell lines), a concentration that readily triggered apoptosis in non-caspase-9-blocked H12.1 cells but not in resistant 1411HP cells. As shown in Fig. 6A, abrogation of caspase-9 activity in H12.1 cells also resulted in a lack of activation of caspase-2 and caspase-3. This result suggests that caspase-9 acts as an upstream caspase in the cascade in response to cisplatin treatment. After blockage of caspase-9, H12.1 cells exhibited chemoresistant characteristics similar to 1411HP cells in the SRB cytotoxicity assay (Fig. 6B). However, treatment of caspase-9-blocked H12.1 cells with 10 μM cisplatin (the IC90 of 1411HP) readily induced apoptosis (data not shown). Interestingly, the adherent cell fraction of caspase-9-blocked H12.1 showed a similar pattern of caspase activation as 1411HP because significant activity was only observed for caspase-2 and caspase-3 (Fig. 6A). This demonstrates that inhibition of caspase-9 in sensitive testicular cancer cells could not ultimately block apoptosis but induced a higher cellular threshold for apoptosis so that higher levels of DNA platination were required to initiate the apoptotic cascade. This behavior is indicative of cisplatin resistance. Western blot analysis (Fig. 6C) confirmed that caspase-9 was not cleaved/activated in inhibitor-treated H12.1 cells after treatment with 3 μM cisplatin for 24 h, suggesting that in sensitive cells, caspase-9 is cleaved and activated by autoactivation. Treatment of caspase-9-blocked H12.1 cells with 10 μM cisplatin for 24 h resulted in the cleavage of caspase-9, although no caspase-9 activity could be detected (Fig. 6A). This suggests that caspase-9 was cleaved by a different factor but failed to become enzymatically active in the presence of the inhibitor. Thus, the apoptotic cascade in caspase-9-blocked H12.1 cells was likely initiated by an alternative, caspase-9-independent pathway.
blocked H12.1 with 10 \( / \) cisplatin resistance comparable with the cell line 1411HP (\( B \)). Treatment of caspase-9-

Values represent means similar to 1411HP, significant activity was only observed for caspase-2 and caspase-3 (\( A \)). Inhibition of caspase-9 in sensitive H12.1 cells resulted in a lack of cleavage/activation of caspase-2 and caspase-3. However, it does not prevent apoptosis after treatment with high cisplatin doses, which overrides this block. In addition, blockage of caspase-9 in the sensitive cell line 2102EP treatment with high cisplatin doses, which overrides this block. In addition, blockage of caspase-9 in the sensitive cell line 2102EP induced cisplatin resistance in a similar manner as in H12.1 as assessed by the SRB cytotoxicity assay (Fig. 6A).

**Differential Activation of Caspase-9 and Differential Susceptibility for Apoptosis Is Independent of the Expression of p53, Bcl-2 Family Proteins, and Fas.** Hypersensitivity of human testicular cancer to drug-induced apoptosis has been associated with the prevalence of functionally active p53 and a high Bax:Bcl-2 ratio (22). This was confirmed by several studies, which noted the expression of functional p53 and the absence of Bcl 2 expression in testicular tumor specimen (35, 36). These typical characteristics of testicular cancer cells were observed in the sensitive cell lines 2102EP and H12.1 and also in the resistant cell line 1411HP. As shown in Fig. 7, p53 was up-regulated in both sensitive and resistant cancer cells, even at the time point when differential activation of caspase-9 had already occurred (Figs. 4 and 5). Likewise, all Bcl-2 family proteins were tested and not affected by cisplatin treatment, and no differences in protein expression between sensitive and resistant cells were observed (Fig. 7). Typically, Bcl-2 was not expressed in these cell lines. Thus, our results suggest that failure of activation of caspase-9 and cisplatin resistance in 1411HP are not caused by differences in the expression levels of p53 or Bcl-2 family members.

Induction of FasL and up-regulation of FasR was observed after treatment of different tumors with cytotoxic drugs at therapeutic concentrations and an autocrine suicide or paracrine cell death pathway was suggested. In addition, resistance to FasR-induced apoptosis was associated with cross-resistance to therapeutic concentrations of several chemotherapeutic agents (17). Interestingly, resistant 1411HP cells failed to up-regulate the FasR after cisplatin treatment in contrast to the sensitive cell lines 2102EP and H12.1, whereas the Fas-ligand was expressed by all three cell lines (Fig. 7A). However, elevation of FasR protein levels in 1411HP cells by transfection of a FAS gene containing expression vector (Fig. 7B) failed to induce a higher chemosensitivity in 1411HP (Table 3). This demonstrates that the lack
of up-regulation of FasR in 1411HP is not associated with cisplatin resistance. In addition, cisplatin treatment of the sensitive cell lines was not synergistically affected by cotreatment with an agonistic Fas-R-antibody (data not shown). Thus, cisplatin-induced apoptosis appears to be independent of the Fas-system in testicular cancer cells, and up-regulation of the Fas-receptor has no consequences for chemosensitivity. This finding correlates with the Western blot analysis of the cleavage of caspases (Fig. 4) because caspase-8, which mediates Fas-induced apoptosis, appears to be cleaved more downstream and seems to play no critical role in mediating cisplatin-induced apoptosis. It is noteworthy in this context that in a very recent study, the Fas-signaling inhibitor Fap-1 was shown to be present in all histological variants of germ cell tumors tested (37).

**DISCUSSION**

Studies to unravel the molecular mechanism of chemoresistance in cancer cells may help to design new strategies for cancer treatment. Testicular cancer represents one of few cancer types that are highly sensitive to and curable by chemotherapy. However, in a few cases, resistance to cisplatin-based chemotherapy occurs and patients eventually die from progressive tumor growth. The reason for resistance in testicular cancer cells is unclear, in particular, in view of the high chemosensitivity in general. To date, analysis of various pharmacological and molecular parameters have failed to fully explain this phenomenon. The absence of p53 mutations and a high Bax:Bcl-2 ratio has been associated with the hypersensitivity of testicular cancer cells (22, 35, 36). However, differential cisplatin sensitivity between different testicular cancer cell lines was shown to be independent of either DNA platination, p53 status, or expression of Bcl-2 family proteins (38). Recently published results clearly indicate that sensitivity to apoptosis induction of different testicular cancer cells is independent of the p53 status (39). Likewise, in our testicular cancer model, differential sensitivity to cisplatin was not correlated with either differential DNA platination (Fig. 2) or differential expression of p53 and Bcl-2 family proteins (Fig. 7). Nevertheless, the combination of functionally active p53 and a high Bax:Bcl-2 ratio and perhaps other factors may explain at least, in part, why testicular germ cell tumors are generally more sensitive to chemotherapy than other solid tumors. However, the occurrence of chemoresistance in testicular cancers seems to be independent of these molecular parameters.

Progress has been made in the understanding of the pathways that underlie chemotherapy-induced apoptosis, leading to the realization that the sequential activation of caspases is essential for the apoptotic process. In this study, we investigated the activation of caspases in sensitive and resistant testicular cancer cells and identified a potential mechanism that may confer cisplatin resistance. First, we clearly show that the differences in cisplatin sensitivity correlated closely with the differential induction of apoptosis in our testicular cancer model in vitro and in vivo (Figs. 1 and 3, Table 1). The resistant cell line 1411HP demonstrated decreased susceptibility for apoptosis in response to cisplatin-mediated DNA damage that readily triggered apoptosis in the sensitive cell lines 2102EP and H12.1 (Fig. 2, Table 1). This implies that 1411HP cells have a higher threshold to induce apoptosis that correlates with a higher tolerance for DNA-damage so that a much higher amount of DNA cisplatin adducts is required to initiate the apoptotic cascade. Our in vivo data in nude mice underscore these findings by demonstrating that in H12.1 tumors the substantial occurrence of apoptotic cells was associated with tumor regression in contrast to 1411HP tumors, which showed neither increased apoptosis nor tumor regression under treatment with clinically relevant concentrations of cisplatin (Fig. 3). Subsequently, we showed that in testicular cancer cells initiation of cisplatin-induced apoptosis is preferentially mediated by caspase-9 and not by caspase-8 and that the resistant testicular cancer cells fail to activate caspase-9 as an initial step of apoptosis (Figs. 4 and 5). This phenomenon was independent of the expression of key regulators of apoptosis such as p53, Bcl-2 family proteins, and the Fas system (Fig. 7). Thus, cisplatin-induced apoptosis in resistant 1411HP cells is mediated by an alternate, caspase-9-independent way. However, this pathway needs a higher amount of DNA damage, i.e., higher concentrations of cisplatin, for its initiation. Blocking of caspase-9 in the sensitive cell lines H12.1 and 2102EP induced a resistance pattern similar to the resistant cell line 1411HP (Fig. 6B). As in 1411HP, the higher threshold for apoptosis after abrogation of caspase-9 could be readily overcome by treatment using the IC_{50} cisplatin doses of the resistant cell line 1411HP, which implies that the apoptotic cascade in the caspase-9-blocked H12.1 was triggered in a caspase-9-independent manner (Fig. 6, A and C). This finding demonstrates the existence of functionally redundant mechanisms of apoptosis initiation after a defined stimulus and that the apoptotic process can be started by an alternate, possibly caspase-2 or caspase-3-dependent way. This alternate pathway, however, requires a higher concentration or longer exposure of cisplatin for its initiation. Therefore, the caspase-9 pathway is not necessarily required for cisplatin-induced apoptosis but is crucial for chemosensitivity, at least in our testicular cancer model. According with this concept is that resistance of 1411HP was associated with resistance to drugs of other substance classes e.g., topotecan, etoposide, docetaxel, vincristine, and gemcitabine in the SRB cytotoxicity assay (data not shown). This confirms the importance of our findings of a failure of activation of caspase-9 and a higher apoptotic threshold within resistant 1411HP cells because caspase-9 is thought to be critical in drug-induced apoptosis, not only in cisplatin-induced apoptosis. Moreover, in our sensitive testicular cancer cells, cleavage of caspase-9 appeared to be the initial step during the induction of apoptosis after treatment with 3 \mu M cisplatin. Thus, activation of caspase-9 is the most important event but certainly not the only step leading to the irreversible point in the apoptotic program. The interaction between caspase-9 and caspase-3 is likely critical in this apoptotic pathway because caspase-3 is the most active and most important caspase in executing the apoptotic program. However, as demonstrated in the resistant 1411HP and in the caspase-9-blocked H12.1, caspase-3 and caspase-2 alone are not activated after treatment with low cisplatin doses. Therefore, active caspase-9 is the trigger that starts the apoptotic program after treatment with low doses of cisplatin. After treatment of resistant cells with high doses of cisplatin, caspase-9 is not required as an apoptotic trigger as it is not active in early apoptotic 1411HP cells and its activity is inhibited in early apoptotic cells of caspase-9-blocked H12.1. Under these conditions, other potential start mechanisms associated with caspase-2 and caspase-3 are likely evoked. Therefore, in our testicular cancer model, activation of caspase-9 is responsible for susceptibility to apoptosis after treatment with low cisplatin doses, whereas after treatment with high cisplatin doses, caspase-9 is not involved in the induction of apoptosis.

Interestingly, the availability of the caspase-9 pathway is conceivably responsible for chemosensitivity in various malignant tumors.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Quantification of apoptotic cell population (percentage of all cells) by flow cytometry using the apoptosis marker Apo 2.7</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td>1411PCR3.1</td>
<td>4</td>
</tr>
<tr>
<td>1411PCR3.1.FAS</td>
<td>3</td>
</tr>
</tbody>
</table>

Apo 2.7 was measured by flow cytometry. Cell lines were treated with 10 \mu M cisplatin for 24 h, washed, and both adherent and floating cells were pooled and processed after 24 h. Apoptotic populations were calculated in percentage of the whole cell population. Values represent the mean of three independent experiments with a SD \leq 10%.
Liu et al. (40) recently published observations similar to our findings in an ovarian cancer model demonstrating that failure to activate caspase-9 induces chemoresistance. Furthermore, other groups have confirmed the critical role of caspase-9 in mediating anticancer drug induced apoptosis (41–43).

Recently, the new group of the inhibitors of apoptosis proteins have gained increased importance in the field of apoptosis research. Interestingly, some of the inhibitors of apoptosis proteins are able to confer resistance to apoptotic cell death by direct inhibition of distinct caspases, including caspase-9 (44). Therefore, they are candidates of interest for additional investigation in our model. Because the pluripotent malignant testicular germ cells can result in tumors of various histological subtypes, it is important to note that 1411HP cells, when maintained as xenograft in nude mice, produce yolk sac elements (24, 45) in contrast to 2102EP and H12.1 cells (45). Mediastinal germ cell tumors, which frequently show yolk sac elements, are often refractory to chemotherapy, relapse more frequently and are associated with poor survival (46). Furthermore, pure mediastinal yolk sac tumors, which are extremely rare, are associated with a very poor prognosis (47). Interestingly, Huddart et al. (7) described a yolk sac tumor cell line, which exhibited similar cisplatin resistance characteristics as in 1411HP. However, whether a failure of activation of caspase-9 is a typical feature of this subtype of germ cell cancer remains to be elucidated.

In conclusion, our results demonstrate the importance of the caspase system for chemosensitivity to cisplatin. In particular, we were able to show that failure of activation of caspase-9 confers cisplatin resistance in human testicular cancer cells by inducing a higher threshold for apoptosis. However, this higher threshold can be overcome by higher cisplatin doses or a longer time of cisplatin exposure, thereby in principle supporting the clinical strategy of high-dose chemotherapy in patients with chemorefractory germ cell tumors (48–50). However, because of the higher toxicity of this approach, targeting the factor responsible for blocking activation of caspase-9 represents a more favorable therapeutic strategy.

Additional studies on caspase pathways and alternate mediators of apoptosis may help to explain the differences in chemosensitivity between different cancer cell types and could lead to molecular strategies to overcome cisplatin resistance in the future.

ACKNOWLEDGMENTS

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REFERENCES


**Announcements**

**MEETING OF THE RADIATION RESEARCH SOCIETY**

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22–24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

**ERRATUM**

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 891; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O₂ consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if M = +0.27 and L = −0.16 and the normal differential is 65 per cent M and 35 per cent L, then

0.65 (+0.27) + 0.35 (−0.16) = +0.12,

a figure identical to the observed +0.12 for normal leukocytes.
Failure of Activation of Caspase-9 Induces a Higher Threshold for Apoptosis and Cisplatin Resistance in Testicular Cancer


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