Sequential X-Irradiation Induced Acquired Resistance to Oxaliplatin but Increased Sensitivity to Cisplatin in Two Human Teratoma Cell Lines In Vitro

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Abstract. Cisplatin (CDDP) and oxaliplatin (OXA-P) are potential therapeutic drugs in the treatment of testicular cancer. However, the emergence of drug resistance has been documented not only in patients after chemotherapy but also subsequently to fractionated X-irradiation. Specific radiation-induced biochemical alterations may play a role in the observed resistance. Since irradiation influences the cellular responses to chemotherapy, this study investigated changes in the expression of key proteins in the regulation of DNA repair and apoptosis subsequent to sequential irradiation. Materials and Methods: Logarithmically growing human teratocarcinoma cell lines 2101 EP and H 12.1 were irradiated (10 fractions of 4 Gy in vitro) to establish the sub-lines 2101 EP/DXR-10 and H 12.1/DXR-10. Radiosensitivity was assayed using a clonogenic survival assay. Drug response was assayed using a sulforhodamine B assay. Expression of p53, PARP, hMSH2 and Fas was detected by Western blotting. Results: Both DXR-10 sub-lines showed a significant increase in sensitivity towards CDDP as compared to their parental cell line, however, there was a concomitant increase in resistance against OXA-P. No significant changes in radiosensitivity between parental and DXR-10 cell lines were observed. In addition, there was an up-regulation of PARP, p53, hMSH2 and Fas in the DXR-10 sub-lines, implicating induced damage tolerance and repair mechanisms following irradiation. Conclusion: These results suggest that radiation preceding chemotherapy might induce resistance to subsequent chemotherapy with oxaliplatin but not to cisplatin. This is a novel observation and, if confirmed, particularly in other tumor types, may have clinical implications.

Studies on the molecular mechanisms of interactions between radiotherapy and chemotherapy in human tumor cells are of potential significance for the combined modality approach in cancer treatment. Amongst the drugs which are frequently combined with radiation, cisplatin (CDDP) and oxaliplatin (OXA-P) are some of the most widely used in the treatment of a variety of solid tumors. Although combined treatment with CDDP or OXA-P and radiation has made a positive impact in the management of several human malignancies, many are not controlled despite this multimodality approach and optimal sequencing of each modality remains to be determined.

Numerous factors have been implicated in modulating CDDP or OXA-P sensitivity. Decreased accumulation and/or increased efflux is one of the most frequent mechanisms of platinum resistance and may be one of the earliest resistance mechanisms to develop in cell lines exposed to CDDP repeatedly (1). Increases in metallothionein, glutathione and/or glutathione-S-transferase or changes in reduced folate metabolism have been reported in many CDDP-resistant cell lines (2). Increased DNA repair activity is also an important mechanism of platinum resistance. In terms of cellular DNA repair, there appears to be little or no specificity for the repair of Pt-DNA adducts with cis-diammine, ethylenediammine(en), or 1,2-diaminocyclohexane (DACH) carrier ligands (3). Recent experiments have suggested that defects in mismatch repair (MMR) can also lead to CDDP resistance, and occurs frequently during the acquisition of CDDP resistance in cell culture and has been implicated in resistance occurring after repeated cycles of CDDP in clinical trials (4). The defect in MMR allows resistant cells to tolerate DNA damage and replicate, instead of undergoing cell-cycle arrest or cell death. Previously, Fink et al. (5) showed that colon carcinoma cell lines either defective in hMLH1 or hMSH2 MMR enzymes are 1.5- to 2-fold more resistant to CDDP, but display little or no resistance to OXA-P. Moreover, it has been shown that MMR

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complexes recognize CDDP diadducts, but not DACH-Pt diadducts in DNA (6), suggesting that this particular resistance mechanism may account for the differential cytotoxicity profile between DACH- and cis-diammine-Pt compounds. The participation of oncogenes and tumor suppressor proteins in platinum resistance has been discussed in a review (7). The p53 protein acts as a tumor suppressor and is an important part of the stress response in cells (8). Activation of this protein can induce different pathways that culminate in cell-cycle arrest or in apoptosis. Loss of p53 activity therefore would allow damaged cells to survive following exposure to platinum compounds.

In a general in vitro study of interactions between a series of clinically useful antitumor drugs and X-irradiation, Hill et al. (9) showed that prior exposure of human tumor cells to X-irradiation results either in the expression of subsequent resistance or collateral sensitivity to CDDP. Furthermore, this group provided evidence that in vitro exposure to fractionated X-irradiation of an apparently repair-deficient teratoma cell line results in the expression of resistance to CDDP associated with enhanced repair and tolerance of CDDP-induced DNA damage (10). Similar results were reported by Dempke et al. (11) who showed that CDDP resistance following exposure of SK-OV-3 cells to fractionated X-irradiation was associated with enhanced repair of CDDP-induced DNA adducts and reduced drug uptake. In contrast, collateral sensitivity to CDDP following X-irradiation of the human ovarian carcinoma cell line JA-T has been found and has been associated with a decreased activity of DNA polymerase β (12).

The processes by which fractionated X-irradiation selects or induces resistance or collateral sensitivity to CDDP remain to be determined. However, since these initial investigations suggested that repair of platinum-DNA adducts can be modulated by X-irradiation, the main objective of this study was to clarify further whether prior radiation can induce collateral sensitivity or resistance to CDDP and OXA-P and, if so, to identify the underlying molecular mechanisms.

Materials and Methods

Cell lines and culture. The parental cell lines 2102 EP and H 21.1 were initially derived from two untreated patients as the primary tumor and classified histologically as teratocarcinomas with yolk sac tumor (13, 14). Cells were maintained as monolayers in RPMI-1640 medium (Biochrom, Germany) supplemented with 10% fetal calf serum (FCS, Biochrom, Germany) and 5% CO₂. CDDP was obtained from Sigma Chemicals (Munich, Germany) and OXA-P was obtained from Sanofi (Berlin, Germany).

The DXR-10 sub-lines were derived by exposing logarithmically growing cultures of 2101 EP and H 12.1 parental cells to ten fractions of 4 Gy (dose reducing survival by ~1 log). The total radiation dose administered (40 Gy) was within the range used in clinical radiation therapy protocols for testicular cancer. The medium was changed between each X-ray fraction and cultures were allowed to repopulate and resume logarithmic growth. Both DXR-10 sub-lines were obtained after a period of 25 weeks in culture.

Routine culture conditions of the DXR-10 sub-lines, once established, were identical to those used for the parental cells, which were also maintained in culture during the period of sub-line development. Population doubling times, cell volumes, and DNA and protein content showed no significant differences between the parental cells and those of the corresponding sub-line, and there was no evidence that any of these properties changed significantly during a continuous culture period of at least six months.

Cytotoxicity assays. All cytotoxicity assays were carried out using the sulforhodamine B (SRB) assay (15). A total of 3,000 cells were seeded into 96-well plates (Falcon, Germany) and allowed to attach overnight. Different concentrations of either CDDP or OXA-P were added for 24 h. Cells were then washed with RPMI-1640 medium without FCS. The plates were incubated with normal RPMI-1640 (plus 10% FCS) for 96 h. After incubation cells were fixed with 10% trichloracetic acid and stained with SRB. Protein bound SRB dye was dissolved in 10 mM Tris and measured with an ELISA reader (SLF-rainbow) at 540 nm. The absorbance for each dose was expressed as a percentage of the control of untreated cells.

Radiosensitivity was measured using the colony-forming assay. Exponentially growing cell cultures were X-irradiated with either 1, 2, 3, or 4 Gy. Subsequently, they were trypsinized and 300 cells/flask were passaged with an excess of growth medium. After a growth period of 14 days medium was removed and cells were fixed with 4% formaldehyde for 20 min. After rinsing with PBS, cells were stained with Giemsa and colonies of more than 50 cells were counted. Dose response plots were created using Sigma plot and SF-2 doses (growth inhibition at 2 Gy) were calculated from the plots.

Measurement of PARP (poly-ADP-ribosyl polymerase), p53, hMSH2, and Fas. Logarithmically growing cells were harvested for Western blotting experiments. A total of 10⁷ cells (parental cell lines and corresponding DXR-10 sub-lines, respectively) were resuspended in 1 ml RIPA lysis buffer (100 mM NaCl, 0.5% Igepal, 0.5% SDS, sodium deoxycholate, 50 mM Tris (pH 8 containing 1 mM PMSF, 0.1 mM DTT and 50 μM protease inhibitor (Sigma Chemicals, Munich, Germany). After centrifugation at 13,000 rpm for 15 min, the protein content of the supernatant was estimated using the Bradford assay (16). Equal amounts of protein (60 μg/lane) were separated in 8% (for hMSH2) or 12% (for p53, PARP, and Fas) SDS-PAGE (45 minutes, 200 V), and were transferred to nitrocellulose membrane (Bio-Rad, Munich, Germany) by electroblotting. Blots were stained with Ponceau S. Blots were pre-blocked in PBS with 0.1% Tween (PBST) and 5% dried nonfat milk for 1 h. The following primary monoclonal mouse IgG antibodies were used for detection: hMSH2: GB-12 (Oncogene Research, Cambridge, USA); p53: Do-7 (Santa Cruz, Santa Cruz, USA); PARP: 4C10-5 (Pharmingen, San Diego, USA); Fas: C-20 (Santa Cruz). The blots were treated with the primary antibodies for 2 h in PBST with 5% dried nonfat milk. Blots were then washed three times with PBST and membranes were thereafter incubated with the secondary polyclonal peroxidase-conjugated anti-mouse IgG antibody (Santa Cruz) in PBST with 1% dried nonfat milk for 1 h and washed again. For detection the enhanced chemiluminescence (ECL) procedure according to the manufacturer’s protocol (Amersham, Braunschweig, Germany) on Hyper-ECL-Film (Amersham).

Statistical analysis. Differences were tested using the unpaired Student’s t-test. P-values <0.05 were considered statistically significant.
Results

Cytotoxicity assays. Dose–response curves for the 2101 EP and H 12.1 parental cell lines and their corresponding DXR-10 sub-lines following 24 h exposure to CDDP or OXA-P are shown in Figure 1. IC_{50} values were interpolated and a comparison (see Table I) shows that both DXR-10 sub-lines were approximately 1.5-fold more resistant to OXA-P than were the parental cell lines, whereas both sub-lines were found to express collateral sensitivity to CDDP (1.5- to 2.3-fold) as compared with the parental cells. In contrast, responses to acute X-irradiation were unaltered (Table I). The data therefore indicate that the X-ray fractionation protocol used resulted in a modified response to CDDP and OXA-P, but not to acute X-irradiation.

Measurements of PARP, p53, hMSH2, and Fas. This study examined the expression of PARP, p53, hMSH2, and Fas by Western blot analysis in 2102 EP, H 12.1, and their corresponding DXR-10 sub-lines. Both parental cell lines expressed the major MMR protein hMSH2, p53, PARP, and Fas. Following fractionated X-irradiation in vitro, an overexpression of these proteins was found in the DXR-10 sub-lines, however, there were no differences between the OXA-P resistant and the CDDP hypersensitive DXR-10 sub-lines (Figure 2).

Discussion

In an attempt to clarify possible interactions between radiation and platinum compounds, in this study we pre-exposed two human teratocarcinoma cell lines (2102 EP and H 12.1) to fractionated X-irradiation in vitro. Subsequent drug exposure of the newly derived DXR-10 sub-lines revealed significantly altered responses to the parental cells, with DXR-10 sub-lines expressing increased sensitivity to CDDP but acquired resistance to OXA-P. Response to acute X-irradiation, however, was unchanged in both sub-lines.
Resistance to OXA-P can be induced not only after exposure of human tumor cells to the drug itself, but also after exposure to X-irradiation. The level of OXA-P resistance (1.5-fold) shown under these experimental conditions is relatively modest, but the effect is reproducibly detectable. Similar low levels of OXA-P have been reported for other human tumor cell lines (Table II) and are considered to be characteristic of platinum coordination complexes and possibly other alkylating agents (17) and may reflect the clinical situation more realistically.

Studies carried out in cell lines of the NCI anticancer drug screening panel comparing OXA-P and other platinum agents have shown that CDDP and OXA-P have different sensitivity profiles, suggesting that the two complexes may have different mechanism(s) of action and/or resistance (18). As opposed to numerous ‘classical’ factors of platinum resistance, however, little is known about decreased MMR, altered apoptosis or the effects of several signaling pathways. This prompted this study to investigate the protein expression of PARP, p53, hMSH2, and Fas in two human teratoma cell lines with different platinum responses following fractionated X-irradiation in vitro. As judged by Western blot analysis, an increase of PARP, p53, hMSH2, and Fas was found in both DXR-sub-lines. It has been postulated that functional MMR complex recognizes CDDP-DNA adducts, but not OXA-P-DNA adducts and that MMR proteins are involved in mediating apoptotic responses to DNA damage (19). Molecular modeling studies have revealed distinct differences between the structures of CDDP- and OXA-P-DNA adducts, supporting this hypothesis (20). In addition, a differential activation of damage response pathways has been shown for CDDP, but not for OXA-P. Other studies have indicated that MMR defects may contribute to increased replicative bypass of CDDP adducts, and loss of MMR leads to tumor cell resistance by desensitizing cells to specific DNA-damaging agents (4). In the present study, an increase of the major MMR protein hMSH2 was found following X-irradiation when compared to the parental cells, suggesting that elevated hMSH2 levels may contribute to the observed collateral CDDP sensitivity in the DXR-10 sub-lines, whereas resistance to OXA-P is not modulated by MMR mechanisms. The MMR proteins are involved in mediating the activation of cell-cycle checkpoints and apoptosis in response to DNA damage (4). An increase of the expression of MMR proteins may therefore result in drug sensitivity directly by enhancing the ability of the cell to detect CDDP-induced DNA damage and activate apoptosis and indirectly by decreasing the mutation rate throughout the genome. The available preclinical data suggest that tumors that contain a significant fraction of cells with elevated MMR protein levels will demonstrate increased responsiveness to specific drugs by increased apoptosis. This is in line with the observed increase of pro-apoptotic proteins (PARP, p53, Fas) in the current study, suggesting that a radiation-induced modulation of the apoptotic threshold may contribute to the demonstrated collateral sensitivity to CDDP in the DXR sub-lines. Further evidence of support that enhanced apoptosis may be involved in the observed collateral sensitivity to CDDP was provided by an earlier published study (21, 22). In this study low-dose fractionated gamma-irradiation (three cycles of 5×2 Gy) induced CDDP resistance in HeLa cells. Drug resistance was associated with a decreased caspase-dependent apoptotic pathway and a downregulated membrane receptor CD95 (APO-1/Fas). Since the response to acute X-irradiation was also unchanged in this experimental model, the results suggest that changes in the activation of the caspase-dependent signaling cascade are involved in the death pathway initiated by CDDP but not by radiation damage.

The observation that fractionated X-irradiation can induce resistance to OXA-P appears a novel finding, however, the underlying molecular mechanisms are far from clear. OXA-P has been demonstrated to differ in some mechanisms associated with the development of CDDP resistance. Compared with CDDP-conditioned cells, deficiencies in MMR and increases in replicative bypass, which appear to contribute to CDDP resistance, have not been shown to induce a similar resistance to OXA-P (3). OXA-P damages DNA by generating intrastrand and interstrand cross-links similar to those induced by CDDP and repaired with similar efficiency by the nucleotide excision repair machinery (3). However, the bulky diaminocyclogexane group alters the recognition of OXA-P adducts by other downstream mediators of the cellular response (23). For example, MMR deficiency due to mutation of hMLH1 in the colorectal cancer cell line HCT116 confers an increase in replicative bypass of CDDP-DNA adducts and resistance to CDDP-induced cytotoxicity, but does not influence replicative bypass or sensitivity to OXA-P adducts (6). Thus, since MMR deficiency confers resistance to CDDP but not to OXA-P, the observed overexpression of the major MMR protein hMSH2 would not alter OXA-P sensitivity.

Sharp et al. (24) provided the first evidence that OXA-P resistance in a human ovarian carcinoma cell line was

### Table I. Cytotoxicity assay data on 2101 EP, 2102 EP/DXR-10, H12.1, and H12.1/DXR-10 cells (SF-2: survival at 2 Gy).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>SF-2 (%)</th>
<th>IC50 CDDP</th>
<th>IC50 OXA-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>2101 EP</td>
<td>26±7</td>
<td>0.3±0.03</td>
<td>0.21±0.01</td>
</tr>
<tr>
<td>2102 EP/DXR-10</td>
<td>25±3</td>
<td>0.2±0.02</td>
<td>0.31±0.04</td>
</tr>
<tr>
<td>P-value</td>
<td>&gt;0.1</td>
<td>&lt;0.01</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>H 12.1</td>
<td>31±1</td>
<td>0.42±0.1</td>
<td>0.26±0.01</td>
</tr>
<tr>
<td>H 12.1/DXR-10</td>
<td>27±3</td>
<td>0.18±0.01</td>
<td>0.4±0.02</td>
</tr>
<tr>
<td>P-value</td>
<td>&gt;0.1</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

*Cells were exposed to drug for 24 hours and results derived from full dose–response curves were expressed in μM (mean value±SE of triplicate assays of 2-3 separate experiments). SF-2 values are expressed as percentage of control cells.
associated with a \( p53 \) mutation (phe172). In A2780 cells harboring this particular \( p53 \) mutation and loss of \( hMLH1 \), 5-fold resistance to CDDP was observed, but only 1.7-fold resistance to OXA-P. Re-introduction of \( hMLH1 \) into these cells caused no significant change in the sensitivity to CDDP or OXA-P, suggesting that \( p53 \) mutation might be critical for the development of OXA-P resistance. In this regard, it is conceivable that prior exposure of the human teratoma cell lines used in the current study to fractionated X-irradiation resulted in \( p53 \) mutations. This in turn would not be detectable by Western blot analysis since the Do-7 antibody used cannot distinguish between wild-type and mutated \( p53 \) proteins. Since a recently published paper (25) provided conclusive evidence that \( OXA-P \) resistance in the human colorectal carcinoma cell line HCT116 was strongly correlated with down-regulation of \( p53 \), with HCT116 \( p53 \) null cells being the most resistant clones, the possibility remains that the observed \( OXA-P \) resistance in the DXR-10 sub-lines used in the current study may be linked to a functional defective \( p53 \) protein. Furthermore, it has been shown that the \( p53 \)- and Fas/PARP-dependent apoptotic response to OXA-P might be suppressed by concurrent expression of BCL-XL (25). Based on this model, it is conceivable that OXA-P induced BCL-XL expression contributes to the protection of DXR-cells from \( p53 \)-mediated apoptosis following OXA-P exposure and thereby conferred resistance to OXA-P suggesting that the critical determinant of apoptosis is the ratio between pro-apoptotic and anti-apoptotic BCL-2 family members rather than their absolute expression levels (26). Despite this proposal, several other ‘classic’ biochemical mechanisms (e.g. drug uptake, enhanced DNA repair) may also contribute to the observed \( OXA-P \) resistance in the DXR-10 sub-lines used in the current study, and studies are currently underway to further investigate these mechanisms.

In summary, this study shows that \textit{in vitro} exposure of two human teratoma cell lines to fractionated X-irradiation results in the expression of hypersensitivity to CDDP but resistance to \( OXA-P \). The demonstrated ability of fractionated X-irradiation to induce resistance to OXA-P appears a novel observation, which, if confirmed, may have clinical implications.

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**Table II. Comparison of resistance mechanisms in published oxaliplatin resistant cell lines (MEDLINE search from 1996-2008).**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Resistance factor (fold)</th>
<th>Mechanism(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovary (A2780)</td>
<td>8-12</td>
<td>Increased ( \gamma )-glutamyl-transpeptidase (( \rightarrow ) GSH ↑)</td>
<td>El-akawi \textit{et al.} (1996) (27)</td>
</tr>
<tr>
<td>Colon (HCT116, HT29);</td>
<td>3-15.8</td>
<td>( p53 ) Mutation (phe172); Loss of ( hMLH1 )</td>
<td>Sharp \textit{et al.} (2002) (24)</td>
</tr>
<tr>
<td>Colon (various cell lines)</td>
<td>3.1-7.6</td>
<td>Decreased oxaliplatin uptake; decreased adduct formation</td>
<td>Mishima \textit{et al.} (2002) (30)</td>
</tr>
<tr>
<td>Lung (H69)</td>
<td>2</td>
<td>Loss of chromosomal segments</td>
<td>Stordal \textit{et al.} (2006) (31)</td>
</tr>
<tr>
<td>Testis (2102EP)</td>
<td>1.6-1.7</td>
<td>Decreased oxaliplatin uptake</td>
<td>Rennicke \textit{et al.} (2005) (33)</td>
</tr>
<tr>
<td>Testis (2102EP, H12.1)</td>
<td>1.5</td>
<td>Reduced apoptosis (?)</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Figure 2. Expression of the proteins PARP, \( p53 \), \( hMSH2 \), and Fas without drug exposure in the H 12.1, H12.1/DXR-10, 2102 EP, and 2102/EP/DXR-10 cell lines: (a) PARP (lower band 85 kDa, upper band 116 kDa), (b) \( p53 \), (c) \( hMSH2 \) (102 kDa), (d) Fas (lower band 40 kDa; upper band 48 kDa).**
References


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