Resistance Patterns between \textit{cis}-Diaminedichloroplatinum(II) and Ionizing Radiation


Laboratory of Cancer Research and Clinical Oncology, Antwerp University, B-2610 Wilrijk, Belgium. C. T. Coughlin and R. C. Richmond, Semin. Oncol., 16: 31-43, 1989). To determine whether cross-resistance patterns between both cytotoxic approaches exist, resistance against CDDP and ionizing radiation was induced separately in human ovarian cancer cells in a cross-over design. Subsequently sensitivity changes were determined for both treatment modalities.

CDDP resistance was induced previously (P. J. Kuppen et al., Cancer Res., 48: 3355-3359, 1988), and resistant cells were grown at three different levels of CDDP: 0 ng/ml; 250 ng/ml; and 500 ng/ml. Resistance with resistance factor (RF) 3.4 to 5.1 proved to be stable, since withdrawal of CDDP pressure for at least 6 mo did not alter resistance patterns. CDDP-resistant cells also demonstrated stable resistance against ionizing radiation, with RF ranging from 1.7 to 2.0. The resistance patterns could not be explained by differences in growth kinetics and DNA content.

Resistance to ionizing radiation was induced in the same human ovarian cancer cells as used for CDDP resistance studies. Exposure with 1.5 Gy of intermittent irradiation during 6 mo, at time intervals of 48 h, resulted in cells which were able to grow under chronic ionizing radiation pressure. RF was 2.0; the resistance was lost after 6 mo of culturing without ionizing radiation pressure. With intermittent radiation doses of 0.5 and 1.0 Gy, no significant resistance could be induced.

Cells intermittently exposed to 0.5, 1.0, and 1.5 Gy during 6 mo demonstrated increased sensitivity to CDDP, with 0.22 < RF < 0.43. Increased sensitivity was associated with proportionally increased formation of the platinum-DNA adducts. Differences in sensitivity for both ionizing radiation and CDDP were lost after 6 mo of culturing without radiation pressure; therefore, resistance toward ionizing radiation and, likewise, the increased sensitivity to CDDP, were judged to be unstable.

In conclusion, data of the present study demonstrated that development of stable resistance to CDDP is associated with development of stable resistance to ionizing radiation in human ovarian cancer. Contrastingly, increased sensitivity to CDDP was found when resistance against irradiation was induced in the same cells.

INTRODUCTION

CDDP\textsuperscript{3} and ionizing radiation are two cytotoxic treatment modalities that have demonstrated effectiveness in the treatment of various malignancies, such as ovarian and testicular cancer. CDDP and ionizing radiation are currently used either alone or in combination with each other. Firm recommendations on the sequence of irradiation and CDDP treatment cannot be given at the moment, which might be due to a lack of data of the development of resistance against CDDP and/or ionizing radiation. Data of clinical studies suggest that cross-resistance between irradiation and CDDP occurs commonly in human tumors (1-3). To the contrary, CDDP has also been shown to act as a radiosensitizer in both experimental and clinical studies (4).

Mechanisms operating in cancer cells resistant to one or both treatment modalities have not been well documented thus far. In general, resistance against CDDP has been studied with cells selected upon repeated CDDP exposure (5). Sensitivity toward CDDP and ionizing radiation was determined in an alternative way by Schwartz et al. (6). Ten cell lines were obtained from human biopsy material and, after 10 early passages, sensitivity toward CDDP and ionizing radiation was evaluated and compared. Cross-resistance was observed in several cell lines (6).

Data of these studies gave information on the development of resistance patterns to CDDP and, sparingly, on underlying mechanisms. Consequences for efficacy of radiotherapy could be estimated, but no firm conclusions could be drawn.

A different approach has been followed in the present study. Resistance toward CDDP and irradiation was induced separately in the same cell line, i.e., human ovarian cancer cells, by chronic cytotoxic exposure. Consequences for both CDDP and ionizing radiation were established in a cross-over design as depicted in Fig. 1.

MATERIALS AND METHODS

Cell Lines

Human ovarian cancer cells originating from COV413.B (5) were used. The cells were cultivated in tissue culture flasks (Falcon) and grew in a monolayer when maintained in Dulbecco’s modified Eagle’s medium (Gibco supplemented with 10% fetal calf serum (Gibco BRL), aspartic acid, and glutamic acid. They were cultured without any antibiotics and were regularly tested for \textit{Mycoplasma} infection. The cultures were maintained at 37°C in a humidified atmosphere of CO\textsubscript{2}/air (5%/95%). The cells were then designated AOVc-0.

CDDP resistance was obtained in AOVc-0 cells by continuous exposure to different concentrations of CDDP as described by Kuppen et al. (5). CDDP-resistant cells were cultured in three different ways: one was CDDP resistant and was grown without CDDP (AOvC-CDDP/RO); the second was able to grow at a CDDP concentration of 250 ng/ml (0.83 μM) (AOVc-CDDP/250); and the third at 500 ng/ml (1.67 μM) (AOVc-CDDP/500).

For radiation sensitivity studies, AOVc-0 were exposed every 48 h to radiation doses of 0.5, 1.0, or 1.5 Gy during 6 mo (AOVc-IR/0.5, AOVc-IR/1.0, or AOVc-IR/1.5, respectively). The irradiation was performed at 1.47 Gy/min with \textit{Co} \textsuperscript{60} γ-rays, in full build-up conditions by exposition of a 0.5 MMMA (perspex/plaxi). The dosimetry was at beam axis with an ionization chamber. Irradiated cells were also cultivated another 6 mo without irradiation (AOVc-IR/0).

Cytotoxic Assay

The survival curves for irradiation or drug exposure at a 24-h interval of previous exposure were determined by the MTT assay (7-11), slightly modified in our laboratory. This semiautomated growth

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The abbreviations used are: CDDP, \textit{cis}-diaminedichloroplatinum(II); ID\textsubscript{50}, 50% inhibiting dose; RF, resistance factor; Pt-G, Pt(NH\textsubscript{3})\textsubscript{2}-d(GMP); Pt-Ag, \textit{cis}-Pt(NH\textsubscript{3})\textsubscript{2}-d(GpApG); Pt-GG, \textit{cis}-Pt(NH\textsubscript{3})\textsubscript{2}-d(GpGpG); G-Pt-G, \textit{cis}-Pt(NH\textsubscript{3})\textsubscript{2}-d(GpGpG); ANOVA, analysis of variance; MTT, (3-[4,5-dimethylthiazol-2-yl][2,5-diphenyltetrazolium]) bromide, C\textsubscript{4}H\textsubscript{4}N\textsubscript{3}SBr.
assay quantitates the overall number of surviving cells at a given time after exposure to cytotoxic drugs or irradiation.

Sample Pretreatment for Assessment of Cytotoxic Activity of Ionizing Radiation. Cell suspensions were obtained by trypsinization up to 100,000 cells/ml, and 10,000 cells/well were seeded in 48-well tissue culture clusters (Costar). Each well was filled with 0.5 ml of the culture medium. The plates were irradiated at room temperature with 60Co γ-rays (Theratron 780) at a dose rate of 147 Gy/min. Doses administered were 0, 1, 2, 3, 4, 5, 6, 8, and 10 Gy. The plates were placed in the incubator immediately after irradiation. Incubation was performed for 7 days without refeeding.

Sample Pretreatment for Assessment of Cytotoxic Activity of CDDP. Cells were exposed to different CDDP concentrations (0, 2.5, 5.0, 10.0, 20.0, and 50.0 μM) and to different exposure times according to data of previous studies (5, 12, 13). Suspensions of 200,000 cells/ml were made, and 20,000 cells were seeded in each well of the 48-well plates. Each well was filled with 0.5 ml of the culture medium. Cells were incubated at 37°C for 4 days and without refeeding.

Determination of Dose-Response Curves for Both CDDP and Ionizing Radiation. Data acquisition was performed by a uniform procedure. After the incubation period, 300 μl of a solution of 2 mg/ml of MTT were added to each well and incubated for 4 h at 37°C in a humidified atmosphere with 5% CO2. The medium was aspirated with a Pasteur pipet. Formazan crystals were solubilized with 250 μl of dimethyl sulfoxide (Janssen Chimica). The amount of blue formazan product was measured spectrophotometrically at a wavelength of 570 nm in a microtiter reader (EAR-400).

Incubation periods were chosen to meet the essential conditions of the MTT assay as described previously (7–9).

Of each cell line, 10 to 12 wells per survival point were plated, and each experiment was performed in triplicate. Calculations were made of the mean ± SD. Sensitivity for both treatments was described by the ID50 in order to facilitate comparisons. The RF was calculated as the ratio of the ID50 of the treated cells over the ID50 of the control cells [RF = ID50t/ID50C, in which t is the treated cells, and c is the control cell line]. Values denoted as 0 < RF < 1 indicate, therefore, increased sensitivity. When RF > 1, change of sensitivity was scored as “resistance.” Data analysis included comparisons between the percentage of survival, per dose of CDDP (μM) or per unit of ionizing radiation (Gy) used between cell lines tested, and was performed by one-way ANOVA with Scheffe’s procedure for multiple comparisons. For comparisons between the whole-dose survival curves of the cell lines of interest, the Friedman two-way ANOVA test was used. For all tests P < 0.05 was taken as the level of significance.

Determination of CDDP-DNA Adducts. DNAs were isolated and chromatographed after enzymatic digestion to the unmodified mononucleotides dCMP, dAMP, dTMP, and dGMP and platinum-containing (oligo)nucleotides (14): Pt-G, derived from CDDP monofunctionally bound to guanine; Pt-AG and Pt-GG, from intranstrand cross-links on neighboring bases in sequences pApG and pGpG, respectively; and G-Pt-G, from intrastrand cross-links on two guanines separated by one or more bases and/or from interstand cross-links on guanines in opposite strands of DNA (15). The quantitation of the platinum products, present at identified positions in the column eluate, was performed with immunochemical techniques by the use of specific antisera (15, 16). In this method, the dilutions of the fractions giving 50% inhibition of antibody binding in the competitive enzyme-linked immunosorbent assay were determined and used to calculate the amounts of CDDP-DNA digestion products (15, 16).

RESULTS
AOvC cells are ovarian cancer cells with a doubling time of 24 h under the conditions described. All lines used grew stable with respect to morphology, doubling time, and DNA content when used for these experiments. Resistance against CDDP and ionizing radiation was induced separately in the same cell line, and sensitivity changes for both cytotoxic agents were determined.

Data of the efficacy of CDDP and irradiation obtained with the MTT assay are given in Fig. 2.

CDDP concentration survival curves for the CDDP-resistant cells (AOvC-CDDP/R0, AOV-C-CDDP/R250, and AOV-C-CDDP/R500) and the control (AOvC-0) are presented in Fig. 2A. Survival of control cells rapidly declined to 10% in a concentration range between 20 and 70 μM CDDP, whereas the 10% survival point of CDDP-resistant lines could not be attained at 300 μM. Data of RF reflect resistance against CDDP: 3.4 for AOV-C-CDDP/R250; 3.6 for AOV-C-CDDP/R500; and 5.1 for AOV-C-CDDP/R0. Remarkably, the AOV-C-CDDP/R0 line was less sensitive than all other cell lines to CDDP at the 50% survival level. The significance was lost at 300 μM (15% survival). Thus, withdrawal of CDDP exposure to CDDP-resistant cell lines did, at least, not result in a loss of CDDP resistance. CDDP resistance in AOV-C-CDDP/R0, AOV-C-CDDP/R250, and AOV-C-CDDP/R500 cell lines is therefore judged as stable resistance.

Radiation survival curves for CDDP-resistant cell lines are shown in Fig. 2B. AOV-C-CDDP/R0, AOV-C-CDDP/R250, and AOV-C-CDDP/R500 cells proved to be less sensitive to irradiation than the parental line, i.e., the AOV-C-0 cells. RFs for ionizing radiation of AOV-C-CDDP/R0, AOV-C-CDDP/R250, and AOV-C-CDDP/R500 lines compared with the AOV-C-0 line were 1.7, 1.8, and 2.0, respectively. Differences between resistant cell lines reached the level of significance beyond 8 Gy only (ANOVA, P < 0.05). At the ID50 level CDDP-resistant cell lines demonstrated similar sensitivity to ionizing radiation. At 3.0 Gy, the first deviation of the control curve became evident. Forty % of AOV-C-0 cells were killed, whereas cell kill was 20% in the CDDP-resistant lines. At a dose of 10 Gy, survival of CDDP-resistant cell lines was still 25% or even more. Decreased radiosensitivity in CDDP-resistant cells has been demonstrated to be stable too, since AOV-C-CDDP/R0 cells demonstrated similar radiosensitivity patterns as AOV-C-CDDP/R250 and AOV-C-CDDP/R500 cell lines. Thus, stable cross-resistance between CDDP and ionizing radiation has been clearly demonstrated in those cells in which CDDP resistance has been induced by continuous CDDP exposure.

Radiation survival curves for the cells exposed every 48 h to irradiation (AOvC-IR/0.5, AOV-C-IR/1.0, and AOV-C-IR/1.5) are presented in Fig. 2C. The survival curve of AOV-C-IR/1.5 cells deviated markedly from other curves at relatively low doses (3.0 Gy) and demonstrated a 25% survival at 10 Gy. The RF of AOV-C-IR/1.5 cells was 2.0; the cells were significantly less sensitive to ionizing radiation between 3 and 10 Gy versus all
cell lines. RFs of AOvC-IR/0.5 and AOvC-IR/1.0 (1.0 and 1.2, respectively) were not significantly different from those of control cells. Radiation survival curves of AOvC-IR/1.5 cells demonstrated loss of resistance toward ionizing radiation 6 mo after withdrawal of ionizing radiation pressure at a level of 1.5 Gy/48 h (RF-1.1; Fig. 2D). Therefore, resistance against ionizing radiation was judged to be unstable.

CDDP concentration survival curves of AOvC-IR/0.5, AOvC-IR/1.0, and AOvC-IR/1.5 versus control cells are presented in Fig. 2E. AOvC-IR/0.5, AOvC-IR/1.0, and AOvC-IR/1.5 cells were significantly more sensitive to CDDP compared with the control line (P < 0.05, ANOVA and Friedman analysis). RFs were less than 1.0 (0.29 for AOvC-IR/0.5, 0.22 for AOvC-IR/1.0, and 0.43 for AOvC-IR/1.5, respectively), which was judged as increased sensitivity. Differences of AOvC-IR/0.5, AOvC-IR/1.0, and AOvC-IR/1.5 cell lines for CDDP sensitivity were not significant when tested for the whole curves (Friedman analysis). However, irradiated pretreatments demonstrated significantly increased, radiation dose-dependent sensitivity toward CDDP in the range of 5 to 10 μM CDDP (Fig. 2E). The increased sensitivity to CDDP upon repeated exposure to ionizing radiation of AOvC-IR/0.5 and AOvC-IR/1.0 was clearly associated with proportionally increased formation of platinum-DNA adducts (Table 1).

Increased sensitivity for CDDP of the AOvC-IR/0.5, AOvC-IR/1.0, and AOvC-IR/1.5 cells was lost when cells were cultured 6 mo without radiation pressure; RFs were close to 1.0 then (1.1, 1.0, and 1.1, respectively) (Fig. 2F). Thus, resistance against irradiation in cells which were chronically exposed to irradiation and simultaneously showing increased sensitivity toward CDDP was judged as a transient, unstable change of sensitivity.

#### Table 1. Pt-DNA adducts in human ovarian tumor cells with decreased sensitivity to ionizing radiation because of chronic exposure with 0.5 Gy/48 h (AOvC-IR/0.5) and 1.0 Gy/48 h (AOvC-IR/1.0) versus control (AOvC-0)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pt-G</th>
<th>Pt-AG</th>
<th>Pt-GG</th>
<th>G-Pt-G</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOvC-0</td>
<td>4.2 ± 0.3<em>a (5)</em></td>
<td>12 ± 0.14</td>
<td>47 ± 13</td>
<td>21 ± 3</td>
<td>84</td>
</tr>
<tr>
<td>AOvC-IR/0.5</td>
<td>7.9 ± 1.3 (5)</td>
<td>27 ± 1</td>
<td>96 ± 15</td>
<td>36 ± 0</td>
<td>167</td>
</tr>
<tr>
<td>AOvC-IR/1.0</td>
<td>12.2 ± 1.8 (6)</td>
<td>35 ± 1</td>
<td>117 ± 36</td>
<td>52 ± 3</td>
<td>216</td>
</tr>
</tbody>
</table>

*a Mean ± SD.

*b Numbers in parentheses, percentage.
DISCUSSION

CDDP is increasingly used in combination with irradiation (12), and the effect of the combination of both treatment modalities varies between different tumors and different investigations (17-19). In normal tissue the effects are, to a large extent, explained by independent cell kill by each agent (17). CDDP-induced biochemical alterations in the cell are known to be complex (6, 20, 21). Which of the processes is involved in interactions with irradiation is mainly speculative at the present time. Moreover, investigations on the interaction between CDDP and cellular repair processes upon irradiation are complex, since different DNA repair processes for the two agents are involved (17). The relation between CDDP and radiation resistance in vitro, as demonstrated in the present study, is particularly interesting, having the results of clinical trials with CDDP regimens and radiotherapy in mind. Significant correlations between the response to CDDP and the subsequent response to ionizing radiation have been reported in patients receiving CDDP-based regimens followed by radiotherapy (1, 2, 13, 17, 22-25). A strong correlation between resistance to CDDP and radiotherapy has also been demonstrated. Ensley et al. (2) demonstrated a strong correlation between CDDP resistance and irradiation resistance in head and neck tumors.

Resistance to chemotherapy agents can be governed at both the cellular and the microenvironmental level. Microenvironmental factors that might lead to drug resistance include reduced blood supply with decreased drug delivery, diminished cellular metabolism, and a diminished growth fraction (26). At the cellular level it is shown that CDDP induces DNA-protein cross-links, DNA interstrand cross-links, DNA intrastrand cross-links, and monofunctional adducts (6, 20, 21). Resistance may be based on an altered DNA repair system (6). Kuppen et al. (5) induced resistance to CDDP in a human ovarian cancer cell line by continuous exposure of parental lines to increasing CDDP concentrations in the culture medium. Resistance to CDDP was in part related to decreased amounts of platinum in the resistant cells. Other studies showed resistant cell lines with higher glutathione levels and DNA repair capacity (27). Teicher et al. (28) reported higher glutathione transferase activity and decreased DNA cross-links in their resistant cell line.

In vitro resistance to irradiation can be related to decreased blood supply with resulting hypoxia. Ionizing radiation, however, induces DNA lesions and, therefore, radiation resistance in vitro might occur at the cellular level because of, e.g., an enhanced rate of rejoining DNA double-strand breaks (6, 29, 30). The latter mechanism is important for variations in radiation sensitivity as have been observed between cell lines treated in vitro (31). Some other investigators could indeed demonstrate radiation resistance in mammalian cells following continuous irradiation (32, 33).

Wallner and Li (29) demonstrated that CDDP resistance in fibroblasts does not necessarily confer cross-resistance to irradiation at the cellular level. Cross-resistance between CDDP and irradiation was suggested to be the result of changes in the tumor microenvironment, rather than a common alteration at the cellular level. Louie et al. (34) reported simultaneous acquisition of drug and radiation resistance after exposure of a human ovarian cell line to CDDP. This supports the possibility of cellular cross-resistance between the two treatment modalities. In in vitro studies with various human cell lines, Schwartz et al. (6) found a clear cross-resistance between CDDP and irradiation with various human cell lines; repair for both agents shares a common pathway, and therefore he concluded that resistance to these two agents might reflect DNA repair alterations.

In addition to previous data, we have found a one-way cross-resistance between CDDP and ionizing radiation in CDDP-resistant human ovarian cancer cell lines derived from the parental cell line AOV-C-0. The resistance was stable. On the other hand, induced, unstable resistance to irradiation did not result in cross-resistance to CDDP. To the contrary, the radiation-resistant cells were even found to be more sensitive to CDDP than was the parental line. This phenomenon, also, was not stable.

Resistance to CDDP and ionizing radiation and cross-resistance could not be explained by alterations in cell growth kinetics or DNA ploidy. The possible alterations in cell growth kinetics were determined by the MTT assay. Growth curves of the different cell lines were performed; no difference in growth rate could be found. Data of DNA ploidy measurements and DNA distribution analysis acquired by flow cytometry, according to the method of Vindelöv et al. (35), gave no clear-cut explanation for the resistance patterns observed. The AOV-C-0 cells were characterized as diploid. CDDP-resistant cells had a DNA index in a range of 1.76 to 1.84. This might reflect selective growth of cells under CDDP pressure. All cells chronically exposed to irradiation had a DNA index of 1.16. Therefore, these findings do not explain radiation resistance observed after chronic radiation pressure of the AOV-C-IR/1.5 cells only. Cells exposed to CDDP showed an increased G₂-M-phase fraction, because of the CDDP-induced mitotic arrest. The G₂-M phase is the most sensitive phase for irradiation (36), and therefore an increased G₂-M phase cannot explain the cross-resistance that has been found in our experiments. Additionally, cells continuously exposed to irradiation showed both a pronounced S phase and G₂-M phase, where cells in the S phase have been demonstrated to be less sensitive for irradiation (36). The latter might reflect a partial cell cycle synchronization after chronic exposure to irradiation. This might in part explain increased sensitivity to CDDP.

Increased sensitivity to CDDP upon repeated exposure to ionizing radiation (AOvC-IR/0.5 and AOV-C-IR/1.0) was clearly associated with proportionally increased formation of platinum-DNA adducts. This is, in part, in contrast with the findings of Hill et al. (37), where increased formation of DNA-adducts was counteracted by increased repair. Therefore, the total amount of adducts did not correlate with drug sensitivity (37). In our study, total adduct formation was increased up to 2.5-fold (AOvC-0, 84 fmol/μg, versus AOV-C-IR/0.5, 167 fmol/μg, and AOV-C-IR/1.0, 216 fmol/μg of DNA), but a prevalence for a specific adduct could not be demonstrated. In all cell lines, Pt-G was 5 to 6%, Pt-AG was 14 to 16%, G-Pt-G was 22 to 25%, and most dominantly Pt-GG adduct formation was 54 to 56%. These data indicate that increased sensitivity toward CDDP after chronic exposure to irradiation is a quantitative dominated process at the DNA level rather than a qualitatively governed one. The present findings of increased sensitivity can be explained by an increased drug permeability and/or a quantitatively altered interaction of the drug with the target DNA. Our data of adduct formation indeed show that the latter mechanism may be present and that higher intracellular levels of CDDP in cells might be associated with increased adduct formation. On the other hand, increased S phase and G₂-M phase as a consequence of irradiation before exposure may subject ovarian cancer cells to increased CDDP/DNA-adduct formation.

The present data are suggestive for different mechanisms operating in the development of resistance against CDDP and...
ionizing radiation. Sklar (38, 39) demonstrated the involvement of ras in altered sensitivity of ras-transformed NIH 3T3 cells against ionizing radiation and CDDP. McKenna et al. (40) revealed a synergistic effect of the v-myc oncogene with H-ras on the development of radioresistance. Involvement of ras seems more likely in cells which developed stable resistance against CDDP and ionizing radiation rather than in cells which developed a transient resistance against ionizing radiation and increased sensitivity to CDDP. Ongoing studies of ras and myc expression in cells of interest might provide definite conclusions on the underlying mechanisms of the present findings.

In conclusion, induction of CDDP resistance in a human ovarian cancer cell line has been shown to result in cross-resistance for ionizing radiation. Induction of resistance against irradiation was possible by means of chronic exposure to radiation. The latter resistance was found to be transient, and cells demonstrated, also temporary, elevated sensitivity to CDDP. Therefore, it can be stated that resistance to CDDP might have unfavorable consequences for ionizing radiation, but the reverse is not true.

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