Combination Photoimmunotherapy and Cisplatin: Effects on Human Ovarian Cancer Ex Vivo

Linda R. Duska, Michael R. Hamblin, Jaimie L. Miller, Tayyaba Hasan

Background: Patients with ovarian cancer that is clinically resistant to cisplatin-based chemotherapy have little hope of a cure of their disease. Photoimmunotherapy, which involves the antibody-targeted delivery of a nontoxic photosensitizer that is activated to a cytotoxic state with visible light, may offer a new treatment option. Phototherapy may be applied intraperitoneally to target disseminated tumor. We tested the hypothesis that this treatment in combination with cisplatin potentiates cytotoxicity in ovarian cancer cell lines and primary cultures of human tumors.

Methods: Five human cancer cell lines (ovarian and breast) and 19 primary cultures were studied. The primary cultures were from solid and ascites tumor samples obtained from 14 patients with ovarian cancer who were undergoing primary surgery. The photosensitizer chlorin $\epsilon_6$ was conjugated to the F(ab')$_2$ fragment of the murine monoclonal antibody OC-125, which is directed against the antigen CA 125. Cytotoxicity was measured by the microculture tetrazolium assay. Treatments consisted of cisplatin alone, photoimmunotherapy alone, and photoimmunotherapy followed by cisplatin. The fractional product method was used to assess synergy in treatment effects. Ex vivo cultured human cells exhibiting 80% or greater survival at cisplatin concentrations of 10 $\mu$M for 24 hours were defined as cisplatin resistant for this study.

Results: When all cell types (cisplatin sensitive and cisplatin resistant) were considered together, combination treatment yielded cytotoxicity that was, on average, 6.9 times (95% confidence interval = 1.86–11.94) greater than that of cisplatin alone (two-sided $P = .023$). Cisplatin-resistant cells showed a synergistic effect of the two treatments (two-sided $P = .044$), while cisplatin-sensitive cells showed an additive effect.

Conclusion: These ex vivo data suggest that platinum resistance in human ovarian cancer cells may be reversible by pretreatment with OC-125-targeted photoimmunotherapy. Further studies are required to confirm the efficacy of this approach in vivo.

The current accepted first-line therapy for advanced ovarian cancer is surgery followed by chemotherapy, which usually consists of a platinum-based regimen (1). Unfortunately, drug-resistant disease frequently recurs, and there is currently no effective salvage treatment available that improves the chance of survival. Therefore, there is a clear need for the development of new strategies for the management of advanced epithelial ovarian cancer.

Photodynamic therapy, an approach to the treatment of neoplasms recently approved by the Federal Drug Administration (2), may offer one such option. In photodynamic therapy, nontoxic photocytotoxic compounds called photosensitizers are accumulated preferentially in malignant tissues (3). Exposure to the appropriate wavelength of light causes phototoxicity by the production of active molecular species such as singlet oxygen (4). Phase II clinical trials using Photofrin® are in progress for the treatment of disseminated intraperitoneal cancer (2). Because ovarian cancer, even in its advanced stages, is almost always limited to the peritoneal cavity, illumination can be restricted to the areas of disease by delivering the light via laparotomy or laparoscopy, thus limiting systemic toxicity. However, the complexity of the peritoneal cavity may require a higher degree of selectivity of the photosensitizer for tumor than is possible with the existing small photosensitizer molecules. This selectivity may be achieved by linking the photosensitizer to a monoclonal antibody (MAb) directed against an ovarian cancer-associated antigen in an approach termed photoimmunotherapy (5–9). Administering the photoimmunoconjugate via the intraperitoneal route as opposed to the intravenous route avoids the problems of uptake of antibodies by the reticuloendothelial system and difficulties in extravasation of the photoimmunoconjugate from the blood vessels in the tumor. This potential for increased selectivity with the MAb should minimize the toxicity to normal tissues, which was a problem in phase I clinical trials (10).

Our laboratory has had a long-standing interest in the preparation and use of MAb-photosensitizer conjugates for photoimmunotherapy (5,6,9,11). A novel series of photoimmunoconjugates with the use of the F(ab')$_2$ fragment of MAb OC-125 and the photosensitizer chlorin $\epsilon_6$ ($\epsilon_6$) has been generated in which the overall charge of the molecule can be manipulated to be either polycationic or polyanionic (12). OC-125 is a murine MAb that recognizes the cell surface antigen CA 125 (13,14), a 1000-kd glycoprotein expressed by 85% of nonmucinous epithelial ovarian cancers (15). The cationic photoimmunoconjugate was found to be more effective in killing ovarian cancer cells than the anionic photoimmunoconjugate in vitro (12) and, in addition, gave superior tumor selectivity when delivered intraperitoneally to a nude mouse xenograft model of human epithelial ovarian cancer (16).

There have been several studies (17–19) on ex vivo chemosensitivity testing that have found an association between the sensitivity of primary cultures established from patients' tumors and their subsequent clinical response. To investigate the potential of the combination of cisplatin (CDDP) and photoimmunotherapy to treat ovarian cancer, we de-

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rived primary cultures from a variety of solid and ascites human ovarian cancers. We compared the effects of individual and combined photoimmunotherapy and CDDP treatments in these primary cultures and in established human cancer cell lines.

**Materials and Methods**

**Cell lines.** NIH: OVCAR-5 and NIH: OVCAR-10 human ovarian cancer cells were purchased from Thomas Hamilton (Fox Chase Cancer Institute, Philadelphia, PA). NIH: OVCAR-3 cells were obtained from the American Type Culture Collection (Manassas, VA). MDA-MB 435 and MDA-MB 231 are human breast cancer cell lines and were provided by Janet Price (The University of Texas M. D. Anderson Cancer Center, Houston). All three OVCAR cell lines were grown in RPMI-1640 medium, while MDA-MB cell lines were grown in Dulbecco’s modified Eagle medium with low glucose. Both media were supplemented with 10% heat-inactivated fetal calf serum, 10% HEPES, penicillin (100 U/mL), and streptomycin (100 μg/mL) and were maintained at 37 °C in an atmosphere of 5% carbon dioxide. For all experiments, cells were grown to 80%–90% confluence and were harvested by use of trypsin–EDTA (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD), and 10,000 cells per well were seeded in 96-well plates and incubated overnight.

**Primary human cancer cell lines.** Solid tumor samples and ascites samples were obtained in a sterile fashion from 14 patients believed to have advanced epithelial ovarian cancer who were undergoing primary cytoreduction surgery at the Massachusetts General Hospital (Boston). At the time the study was initiated, institutional policy did not require informed consent for the use of discarded tissue. Patient information, including age, preoperative serum CA 125, final pathology, and response to tissue. Patient information, including age, preoperative serum CA 125, final pathology, and response to treatment was obtained from the patients' hospital files and their private physicians. Solid tumor samples were obtained from large omental metastases or from the ovarian mass itself. Solid tumor samples were obtained from large omental metastases or from the ovarian mass itself. Solid tumor samples were obtained from large omental metastases or from the ovarian mass itself.

**Photoimmunotherapy and combination treatment.** MAb OC-125 (Fab′)2 was from Centocor, Inc. (Malvern, PA). The conjugation procedure has been described in detail elsewhere (12). Briefly, poly-L-lysine (molecular weight 25,000) was treated with the reduction of substrate 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) to a dark-blue formazan product by mitochondrial dehydrogenases in living cells. Cells were incubated with 100 μL of medium containing 0.5 mg of MTT for 1 hour, followed by aspiration and addition of 100 μL of dimethyl sulfoxide (DMSO) and shaking to dissolve the formazan, and the absorbance was read at 570 nm in the automatic microplate reader. Survival curves were plotted to determine the IC50 (concentration that causes 50% inhibition of growth) for each cell line. Each point on the survival curves represented the mean survival fraction from six wells.

**Statistical analysis.** Because the number of cells that could be obtained from primary cultures of human tumors was limited, dose–response curves could not be derived; thus, isobologram and median effect analysis of potential interaction were not possible. For this reason, fractional product analysis as recommended by Greco et al. (20) was employed. The raw data consisted of mean survival fractions (s.f.) from CDDP and photoimmunotherapy alone and from the combination of CDDP and photoinmunotherapy. Cytotoxic fractions (c.f.) were calculated as 1 – s.f. Bliss synergism was then tested by calculating the fractional product parameter according to the fractional product analysis method of Webb (21). The fractional product value is defined as c.f.[comb]/[c.f. [CDDP] + c.f. [photoimmunotherapy] – (c.f. [CDDP] × c.f. [photoimmunotherapy]). Values greater than 1 indicate Bliss synergism, values approximately equal to 1 indicate additivity, and values less than 1 indicate Bliss antagonism. The fold increase of cytotoxicity of the combination therapy compared with CDDP alone in Table 1 was calculated as c.f.[comb]/c.f.[CDDP]. Patients whose tumors displayed a average survival fraction of 0.8 or greater after treatment with CDDP were considered to be resistant for the purpose of this study. Differences between means were tested for statistical significance by use of two-sided Student’s t test, assuming equal or unequal variances as appropriate. Two-sided P values less than .05 were considered to be statistically significant.

**RESULTS**

CDDP IC50 concentrations were determined for the three ovarian and two breast cancer cell lines. The IC50 for the OVCAR-5 cell line was similar to that for the OVCAR-3 cell line (9.5 and 7.5 μM, respectively). OVCAR-3 was obtained

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from a patient who had a clinical tumor response to platinum-based therapy, while OVCAR-5 was derived from a patient who had not received chemotherapy. The OVCAR-10 cell line is known to be resistant to CDDP (22) (the patient’s disease progressed while being treated with CDDP), and the IC50 is correspondingly high at 29 \( \mu M \).

Similarly, the breast cell lines show resistance to CDDP, with IC 50 values of 17.5 and 26 \( \mu M \).

As a consequence of the above findings, concentrations of CDDP at 10 \( \mu M \) were chosen for cytotoxicity studies. This concentration is believed to be similar to that achievable in vivo (19).

For ex vivo work, a total of 19 solid tumor and/or ascites samples were collected from 14 patients. Patient characteristics are shown in Table 2. In five of these patients, primary cultures were obtained.

### Table 2. Patient characteristics

<table>
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<th>Patient</th>
<th>Age, y</th>
<th>CA 125, U/mL</th>
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<th>Stage†</th>
<th>Clinical platinum resistant</th>
<th>Follow-up time, mo</th>
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*NED = no evidence of disease; NF = patient lost to follow-up; AWD = alive with disease; DOD = dead of disease.
†International Federation of Obstetrics and Gynecology System.
tained from both solid tumor and ascites cells, while only solid tumor in four patients and only ascites in five patients were successfully cultured. CA 125 serum levels were available for all patients. All patients but one (patient N) had tumor and/or ascites collected at original diagnosis and, therefore, had received no previous chemotherapy. Patient N had previously received chemotherapy, and her disease was persistent. Follow-up information was available for 12 of the 14 patients studied. Clinical platinum resistance refers to whether the patient has disease progression or recurrence within 6 months of platinum chemotherapy. Three patients have died of disease, while six are alive with recurrent disease and three are alive with no evidence of disease. In this study, 12 of 14 patients had primary ovarian or peritoneal disease. Two patients had primary advanced endometrial disease on final pathology but were included in the analysis. Histologies of the ovarian and peritoneal primary tumors included 10 serous papillary tumors, one poorly differentiated tumor, and one mucinous tumor. One of the serous papillary tumors was classified as being of “low malignant potential” (serous borderline in Table 2). All patients were treated with primary surgery followed by intravenous platinum-based chemotherapy, with the exception of the one patient with a low malignant potential tumor, who was treated with surgery alone.

Survival fractions after CDDP alone, photoinmunotherapy alone, and the combination of photoinmunotherapy followed by CDDP are shown in Table 1. There was no statistically significant difference between cells given light alone and cells kept in the dark or between cells given CDDP and light and cells given CDDP in the dark. The fold increase in cytotoxicity by the combination (photoinmunotherapy followed by CDDP) over that found with CDDP alone and the fractional product parameter is also shown. The mean survival fraction of the combination was substantially lower than that of either treatment alone, and the mean increase in cytotoxicity was on average 6.9 times (95% confidence interval = 1.86–11.94) greater than the value found with cisplatin alone (two-sided \( P = .023 \)). Survival fractions of CDDP below 0.8 were classified as platinum sensitive, while those with survival fractions greater than 0.8 were classified as platinum resistant. When the data are grouped into plati-

num-sensitive and platinum-resistant categories, the platinum-resistant cells have a higher increase in cytotoxicity of the combination over CDDP alone than the platinum-sensitive cells (12.92 versus 1.81; \( P = .042 \)). Of interest, the survival fraction after photoinmunotherapy alone was statistically significantly lower for the CDDP-sensitive cells compared with the CDDP-resistant cells (0.49 versus 0.80; \( P = .036 \)). The mean value of the fractional product parameter was 1.95 for the resistant cells, which is statistically significantly greater than 1 (\( P = .044 \)) and thus indicates Bliss synergy. The fractional product value for the sensitive cells was 0.91 (not statistically significantly less than 1: \( P = .30 \)), indicating less than Bliss additivity but not antagonism; the fractional product values for resistant and sensitive cells were statistically significantly different (\( P = .028 \)). Fig. 1 graphically shows this synergy, where the actual survival fraction for the combination treatments is compared with the predicted survival fractions calculated for the combination (the product of the two separate survival fractions). For CDDP-sensitive cells, there was no difference between actual and predicted values (\( P = .97 \)); in contrast, for CDDP-resistant cells, the actual survival fraction of the combination was statistically significantly less than the predicted value.

With a view to possible future clinical application of CA 125-directed photoinmunotherapy, it would be very helpful if an association between the serum CA 125 and the response to photoinmunotherapy could be established. However, when the values of cytotoxicity were plotted against the values for serum CA 125, no statistically significant association was seen. Because the CA 125 bound to the cell membrane is responsible for binding the photoimmunoconjugate, studies were carried out to examine the binding of unconjugated OC-125 F(ab’)_2 to the cell surface by indirect immunofluorescence. Positive immunofluorescence could be demonstrated for all cells studied, which was graded from + to ++++ but was not related to the cytotoxicity of photoinmunotherapy (data not shown).

**DISCUSSION**

Our finding that photoinmunotherapy and CDDP in combination show statistically significant synergistic cytotoxicity toward CDDP-resistant cells and additive cytotoxicity toward CDDP-sensitive cells is encouraging. At this time, the most effective chemotherapeutic agents and the recommended first-line treatment following surgical cytoreduction for advanced ovarian cancer are the combination of platinum and paclitaxel chemotherapy...
Unfortunately, some patients’ disease progresses during therapy or recurs fewer than 6 months following completion of cisplatin therapy, thus indicating clinical platinum resistance (25). There is, therefore, great interest in the methods of reversing clinical platinum resistance (26).

In this study, there were variations in the susceptibility of both ovarian cancer cell lines and primary human tumor cultures to photoimmunotherapy alone. The finding in this study of reduced cytotoxicity toward OVCAR-5 compared with OVCAR-3 cells is in agreement with that reported in another study by use of hematoporphyrin-conjugated cationic OC-125 (27) and was attributed to a lower expression of CA 125 on the OVCAR-5 cells compared with that on the OVCAR-3 cells. The MDA-MB breast cancer cell lines are not expected to express CA 125, and the low levels of killing obtained support this. The primary human ovarian tumor cultures are likely to have varying levels of CA 125 expressed on their cell surface, and this may or may not be related to the serum levels of CA 125. The reason for this is that CA 125 is shed from the surface of cancer cells and travels into the serum (28). Thus, although serum CA 125 has been shown to be a good tumor marker for prognostic evaluation (29), it would be necessary to know both the tumor burden and the extent of CA 125 shedding to arrive at relative levels of expression on the tumor cells. The failure to find any consistent relationship between serum CA 125 levels and the extent of photoimmunotherapy-induced cytotoxicity is, therefore, not surprising. The immunofluorescence showed that all of the primary cultures expressed at least some CA 125.

CDDP alone gave a range of killing, as would be expected with the varying degrees of platinum sensitivities found in clinical practice. A convenient way to categorize the cells as sensitive or resistant was to take the mean of the survival fraction after a 24-hour treatment with 10 μM CDDP as the cutoff. Others (19) have adopted different criteria, e.g., 16.6 μM for 1 hour and greater or less than 50% survival. The combination of photoimmunotherapy and CDDP treatments gave substantial increases (mean, 6.8-fold) in cytotoxicity over that obtained with CDDP alone. When the cells were divided into platinum-sensitive and platinum-resistant subgroups, it was found that the increases in cytotoxicity were much higher for the resistant cells (12.9-fold compared with 1.8-fold); in addition, the platinum-resistant cells showed a synergistic effect of the combination, while the platinum-sensitive cells showed an additive effect.

Some insights that might explain this interaction may be gained by reviewing the mechanisms of cellular toxicity involved with CDDP and photodynamic therapy and the factors leading to platinum resistance. The mechanism of CDDP cytotoxicity has been relatively well elucidated. It is thought that the platinum molecule with two functional groups (after intracellular hydrolysis) reacts at the N-7 position on guanine bases and then forms an intrastrand bidentate adduct with a neighboring guanine or adenine base (30). This DNA damage leads to growth arrest and/or apoptosis at the G1 – S or G2 – M cell-cycle checkpoints, depending on the p53 status of the cell (31,32). Mechanisms of platinum resistance, however, have proved harder to unravel (33). It is likely that the cause of platinum resistance is multifactorial (34) and has components of reduced cellular accumulation, increased DNA repair capacity, increased cellular tolerance to DNA adducts, and detoxification by the increased cellular content of glutathione or metallothionein (35). In vitro cell death after photodynamic therapy can occur either by apoptosis or by necrosis (36). The pathway seems to depend on the chemical structure of the photosensitizer and, consequently, on its intracellular localization (37). It has been shown (12) that this cationic photoimmunoconjugate is predominantly localized in lysosomes, where the mode of death is much more likely to be predominantly necrosis, possibly due to spillage of lysosomal enzymes and other toxic moieties. However, there is a possibility of lysosomally localized photosensitizers relocating in the first few seconds of light delivery and possibly gaining access to mitochondria, where they may be considerably more phototoxic (38). It is conceivable that the cellular damage caused by photoimmunotherapy could interact with mechanisms of platinum resistance in several ways. One possibility is that, since the photoimmunoconjugate binds to the antigen located on the plasma membrane, CDDP uptake could be increased because of damage to the gated membrane channel responsible for part of the CDDP uptake (39). Another possibility is that photoimmunotherapy decreased the cellular supply of glutathione, as has been found in other studies (40). However, it is equally possible that photoimmunotherapy could interfere with DNA repair enzymes (41) or produce cell cycle arrest (42), both of which could interact with CDDP to increase cytotoxicity.

Previous work has been done both in vitro and in animal models with photodynamic therapy by use of unconjugated photosensitizers in combination with CDDP. One study (43) generated a photodynamic therapy-resistant variant of the murine RIF-1 cell line and showed that it had mitochondrial alterations and was cross-resistant to CDDP. One study of murine tumors (44) failed to demonstrate an enhanced tumoricidal effect by combining photodynamic therapy with CDDP, while another study of murine tumors (45) using a different photosensitizer and CDDP found an additive effect.

In summary, this study demonstrates that photoimmunotherapy enhances the cytotoxic effects of CDDP on ovarian cancer cells ex vivo, and this enhancement is synergistic when the cells are relatively platinum resistant. These results suggest that photoimmunotherapy in combination with CDDP may be a real alternative treatment in the ongoing fight against ovarian cancer. Photoimmunotherapy could, therefore, be used in conjunction with the current available first-line therapy, surgical cytoreduction, followed by platinum-based chemotherapy. In addition, patients undergoing second-look laparotomy following a complete clinical response to a platinum-based, first-line therapy could undergo photoimmunotherapy following surgery. The treatment could be given in two clinical scenarios, either as a treatment for microscopic residual disease in the case of a positive second look or as consolidation treatment in the case of a negative second look, since 50% of the patients with negative second-look laparatomies will later present with recurrent disease (46). There might also be a role for photoimmunotherapy as salvage therapy for platinum-resistant disease that recurs later in the clinical course. Because of the frequency of this recurrence, there is great interest in methods of reversing both acquired and intrinsic platinum resistance (26).

Further studies are necessary to investigate the mechanism of the interaction, and median effect analysis and isobolograms (20) should be used to define more
closely the synergism of the combination. Trials in the relevant animal model (47) need to be completed before the utility of the approach of photoimmunotherapy in combination with CDDP can be clinically undertaken. Nevertheless, these results with patient material and established cell lines are encouraging because they have the potential to provide new options for patients with advanced ovarian cancer so that, ultimately, we may improve our treatment success rate against this deadly disease.

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NOTES

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