Peptide and Lipid Growth Factors Decrease cis-Diaminedichloroplatinum-induced Cell Death in Human Ovarian Cancer Cells

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ABSTRACT
Growth factors have been demonstrated to regulate the proliferation and viability of a number of cell lineages. Because most drugs used in chemotherapy kill cells through programmed cell death, by the process of apoptosis, we determined whether growth factors, specifically epidermal growth factor (EGF) and lysophosphatidic acid (LPA), which we have demonstrated recently to be a potent growth factor for ovarian cancer cells, would alter the ability of cis-diaminedichloroplatinum (cis-DDP), the most effective chemotherapeutic agent for ovarian cancer, to kill the HEY ovarian cancer cell line. We demonstrate that both EGF and LPA decrease the ability of cis-DDP to kill HEY ovarian cancer cells as assessed by colony-forming cell activity and dye reduction. Morphological changes, DNA release, and electron microscopy suggested that LPA and EGF protect ovarian cancer cells from programmed cell death induced by cis-DDP. Because LPA is present in high levels in ascitic fluid from ovarian cancer patients, and the EGF receptor is expressed by tumor cells from a significant portion of patients where it correlates with prognosis, growth factor modulation of cis-DDP-induced apoptosis may play a role in the poor prognosis associated with ovarian cancer.

INTRODUCTION
Ovarian epithelial carcinoma is the leading cause of death from gynecological malignancy and the fourth leading cause of death from malignancy (1). Current estimates suggest that more than 20,000 women will develop ovarian cancer this year in North America, and that 60% of these women will eventually succumb to their disease (1). The dismal prognosis results from age-dependent and -independent assays (28-31). The growth-stimulatory activity, which we have designated OCAF, is either not present or present at much lower levels in ascites from there has been no significant improvement in cure rates (1-4). Unfortunately, there are no new effective therapies on the horizon, with the only “new” drug for ovarian cancer currently in use (taxol) demonstrating only a slight improvement over platinum-based regimens (3-5).

PCD, through the process of apoptosis, is a mechanism of cell death in which a cell is destroyed by a sequence of energy-dependent events driven from within the cell (6, 7). Morphologically, apoptosis is characterized by condensation of nuclear chromatin, compaction of cytoplasmic organelles, cell shrinkage, and ruffling and blebbing of the plasma membrane (8-10). During the later stages of apoptosis, nuclear DNA is frequently fragmented into oligonucleosome-sized fragments that give rise to characteristic DNA “ladders” upon gel electrophoresis (10-13). However, in a number of systems, oligonucleosomal fragments are not generated, and PCD through apoptosis can occur in the absence of generation of DNA ladders (14-16). To induce cell proliferation, the mechanisms leading to PCD must be bypassed. Thus, many growth factors and cytokines either directly or indirectly activate the biochemical pathways leading to cellular division as well as abrogate intrinsic pathways leading to PCD (17, 18). The role of growth factors in regulating PCD induced by chemotherapeutic agents is not yet clear.

The mainstay of ovarian cancer therapy, cis-DDP, induces apoptosis in Chinese hamster ovary cells (19), the L1210 murine leukemia cell line (20, 21), rat hepatoma cells (22), ovarian cancer cells (23), and the human promyelocytic cell line HL60 as well as the human ovarian cancer cell line SKOV3 (24). Although cis-DDP clearly induces PCD in these lineages as assessed by a number of criteria, cis-DDP-induced apoptosis is frequently not associated with the formation of DNA ladders in ovarian cancer cells (14). Because apoptosis plays a major role in the response to radiation and chemotherapy (19, 25-27), the dismal outcome for ovarian cancer may be the result of failure of cis-DDP and other drugs used in the treatment of ovarian cancer to induce PCD in all tumor cells. Thus, an understanding of the regulation of PCD in ovarian cancer cells may lead to unique, effective therapies for this dismal disease.

Ascites from ovarian cancer patients is a rich source of growth factor activity for ovarian cancer cells in both anchor-age-dependent and -independent assays (28-31). The growth-stimulatory activity, which we have designated OCAF, is either not present or present at much lower levels in ascites from

2 The abbreviations used are: PCD, programmed cell death; EGF, epidermal growth factor; EGFR, EGF receptor; LPA, lysophosphatidic acid; TGF-α, transforming growth factor α; DAPI, 4',6-diamidino-2-phenylindole; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, fetal bovine serum; cis-DDP, cis-diaminedichloroplatinum; OCAF, ovarian cancer-activating factor.
patients with benign diseases such as hepatic cirrhosis and idiopathic ascites (29). Furthermore, ascites from ovarian cancer patients is able to support i.p. growth of human ovarian cancer cells in immunodeficient nude mice (30). We have demonstrated recently that OCAF consists of several forms of LPA (32). Thus, LPA present in ascites may play a role in regulating proliferation of ovarian cancer cells in the patient and thus play a role in the dismal outcome of this disease.

LPA exhibits potent growth factor activity toward fibroblasts (33–35). LPA has been demonstrated to be present in serum; however, this is likely, at least in part, a consequence of platelet activation during the isolation process (36–38) and LPA is likely to be released from growth-factor-stimulated or injured fibroblasts (39). Indeed, the ability of serum to support cell growth appears to be mediated to a major extent by LPA (40–42). LPA has also been demonstrated to induce characteristics associated with the ability to invade (42), suggesting the possibility that OCAF may play a role in both cell proliferation and invasion. Despite extensive studies of the ability of LPA to stimulate proliferation of fibroblasts, the possible role of LPA in the genesis or progression of cancer, particularly in drug resistance, has not been investigated extensively.

EGFR is distributed widely in normal human tissues. Abnormal expression of the EGFR and its ligands has been associated with malignant transformation (43). Indeed, continued expression of the EGFR in ovarian cancer patients is associated with a poor prognosis (43). In support of activation of the EGFR playing a role in ovarian carcinogenesis, the proliferation of some ovarian cancer cell lines in vitro and in vivo is dependent on an intact EGFR signaling cascade (44, 45).

We demonstrate, herein, that both LPA and EGF protect ovarian cancer cells from death induced by cis-DDP. As assessed by morphological and physicochemical criteria, the protective effect appears to occur through inhibition of cis-DDP-induced PCD.

MATERIALS AND METHODS

Cell Lines. The HEY cell line (46) was from Dr. R. Buick (Ontario Cancer Institute, Toronto, Ontario, Canada). HEY cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 2 mM L-glutamine, 10 mM HEPES, 50 μM 2-mercaptoethanol, 10% heat-inactivated FBS (Sigma Chemical Co., St. Louis, MO), and 10 μM gentamicin (Life Technologies, Inc.).

Reagents. MTT and LPA were purchased from Sigma Chemical Co. cis-DDP was from David Bull Laboratories Pty Ltd. (Mulgave, Victoria, Australia). EGF was provided by Allelix Biopharmaceuticals, Inc. (Mississauga, Ontario, Canada). The fluorescent dye DAPI was purchased from Calbiochem (La Jolla, CA).

Cell Treatment. In all experiments in which the cells were exposed to LPA or EGF and cis-DDP, growth factor exposure was for 90 min prior to treatment with cis-DDP. Unless otherwise indicated, all experiments were performed with RPMI with 10% serum.

MTT Staining. Cell viability was assessed essentially as described by Mosmann (47). Briefly, cells were incubated in 100 μl media in 96-well plates with additions as indicated. After incubation, 10 μl of MTT solution (5 mg MTT/ml in H₂O) were added and incubated at 37°C for 4 h. One hundred μl of acid-isopropanol (0.04 N HCl in isopropanol) were added to each well and mixed by pipetting or shaking to dissolve the reduced MTT crystals; the relative cell viability was obtained by scanning with an ELISA reader with a 570-nm filter.

DAPI Assay. This assay was carried out essentially as described by Brunk et al. (48). The fluorescent dye DAPI complexes with DNA to give a product with a fluorescence intensity about 20 times greater than that of the dye alone (49). Cells were treated as specified and harvested, lysed, and pelleted in an Eppendorf centrifuge to separate the DNA fragments from the intact chromat in. The supernatant was then separated from the pellet and transferred to a separate tube. The pellet was then dissolved in GT solution (5 mM guanidine thiocyanate, 0.5% sarcosyl, 25 mM sodium citrate, 100 mM 2-mercaptoethanol). The amount of DNA in both the supernatant and the pellet was determined by addition to a cuvette containing DAPI dye (100 ng/ml). The DNA-induced increase in fluorescence was determined using a Hitachi F4000 spectrofluorimeter. The data are presented as percentage of DNA release, as determined by the following:

% DNA release =

Fluorescence of the supernatant + Fluorescence of the pellet × 100

Colony-forming Assay. This assay was conducted according to that of Sinha et al. (50) and Macpherson and Montagnier (51) as described previously (29). Colony formation in semisolid agarose was assessed after a period of 14 days. Briefly, 60-mm tissue culture dishes were coated with 5 ml of 0.6% agarose, RPMI, and 10% serum; allowed to harden at room temperature for 15 min; and then incubated at 37°C in a humidified CO₂ incubator. Cells were harvested, and a cell suspension was made up of 0.3% agarose. The cells were plated at various dilutions and incubated at 37°C, with twice weekly feedings of 2 ml of 0.3% agarose, RPMI, and 10% serum. Colonies consisting of greater than 20 cells were counted after 14 days.

RESULTS

EGF and LPA Decrease cis-DDP-induced Death of HEY Ovarian Cancer Cells. To mimic in vivo conditions in which patients receive intermittent treatment with cis-DDP, HEY cells were incubated with and without cis-DDP in the presence or absence of EGF or LPA for 24 or 48 h, washed, and the number of viable colony-forming cells assessed by culture in complete media without cis-DDP. The doses of cis-DDP chosen gave a high degree of cell kill and mimicked concentrations achievable in patients wherein cis-DDP frequently induces massive tumor regression. Because HEY ovarian cancer cells have a high colony-forming cell efficiency ranging from 20 to 60%, HEY cells provide a ready assay for cis-DDP-induced cell kill. Importantly, the concentration of LPA utilized is similar to that present in ascites from ovarian cancer patients and similar to that present in the plasma of approximately 40% of ovarian
cancer patients (32). The concentrations of EGF and LPA utilized were assessed in preliminary experiments to be optimal (data not presented). Under the conditions utilized with optimal concentrations of FBS present, EGF and LPA only induce a slight increase in colony-forming cell activity (see Fig. 1 legend). In each experiment presented in Fig. 1, 1.2 × 10^5 colony-forming cell equivalents were incubated with sufficient cis-DDP to give a 5 (Fig. 1A), 3 (Fig. 1B), or 1 (Fig. 1, C and D) log cell kill, which would span the range of what may be achieved by in vivo cis-DDP treatment. As indicated in Fig. 1, both EGF and LPA markedly decreased the ability of cis-DDP to kill HEY ovarian cancer cells regardless of which concentration of cis-DDP was assessed. Indeed, even at doses of cis-DDP (50 μm for 48 h) that decreased colony-forming cell activity from the expected 1.2 × 10^5 colonies to 2 colonies, both LPA and EGF induced an approximate 10-fold increase in cell survival (Fig. 1A).

Because FBS contains a number of growth factors that could interact with EGF and LPA to increase cell survival, the effect of altering FBS concentration on the protective effect of EGF and LPA was assessed. As indicated by a comparison of C and D in Fig. 1, the ability of EGF or LPA to protect HEY ovarian cancer cells from the effects of cis-DDP was not altered significantly by the presence of FBS (10 or 0.5% FBS, respectively).

To provide an assessment of the ability of growth factors to protect ovarian cancer cells from the acute effects of cis-DDP, the effect of EGF and LPA on cis-DDP-induced decreases in cell viability was assessed by a MTT dye conversion assay (47). A 24-h exposure to 10 μm LPA or 25 ng/ml EGF did not consistently alter the number of viable cells present (see Fig. 2 legend). In contrast, incubation of HEY ovarian cancer cells with 50 μm cis-DDP for 24 h induced a decrease in the number of viable cells present (Fig. 2). Once again, both LPA and EGF decreased the effect of cis-DDP on cell viability as assessed by MTT dye conversion and viable cell counts (not presented). Thus, both LPA and EGF decrease the ability of cis-DDP to kill ovarian cancer cells as assessed by residual colony-forming cell activity or as assessed by cell viability.

**EGF and LPA Prevent cis-DDP-induced PCD.** As indicated in Fig. 3A, HEY cells plated for 24 h in RPMI containing 10% serum without cis-DDP appear healthy, and have an epithelioid appearance. In contrast, in the presence of 50 μm of cis-DDP (Fig. 3B), the number of cells present is decreased greatly, and many of the cells exhibit an apoptotic morphology with blebbing (Fig. 3B, arrows) and loss of epithelial morphology. Thus, as indicated by these classical morphological criteria of PCD, almost all of the cells have undergone apoptosis. When cells are incubated with LPA (Fig. 3C) or EGF (Fig. 3D) for 90 min prior to the addition of 50 μm cis-DDP, although a few cells undergo cell death, a large percentage of the cells is protected from cis-DDP-induced cell death and retains normal morphology. cis-DDP-induced morphological changes were associated with marked changes in viable cell number, with 100% of control cells being viable (as assessed by trypan blue exclusion), 15% of cis-DDP-treated cells being viable, 44% of cells treated with cis-DDP in the presence of LPA being viable, and 34% of cells treated with cis-DDP in the presence of EGF being viable.

Transmission electron microscopy confirmed that cis-DDP...
induced morphological changes compatible with cell death through PCD (not presented). Control cells have a healthy appearance, with no condensation of nuclear chromatin and no membrane blebbing. In contrast, after a 24-h exposure to 50 μM cis-DDP, the cell size is decreased with obvious nuclear condensation, changes at the cell surface, and the compaction of cytoplasmic organelles. Many apoptotic bodies were present. Preincubation with LPA or EGF decreases the morphological changes induced by cis-DDP treatment (not presented). Thus, as indicated by light microscopy and transmission electron microscopy, both EGF and LPA protect cells from the action of cis-DDP by preventing apoptosis.

**DNA Fragmentation.** Because apoptosis induced by cis-DDP treatment of ovarian cancer cells is frequently not associated with DNA ladder formation (14–16), we utilized an alternative assay to demonstrate DNA fragmentation (48, 49). As demonstrated in Table 1, control cells show less than 10% fragmentation of DNA. For reasons that are as yet unclear, both LPA and EGF induce a slight but consistent increase in DNA fragmentation. In contrast, cis-DDP induced a marked increase in the amount of DNA fragmentation as indicated by DNA release from the cells (Table 1). Exposure to LPA or EGF before cis-DDP treatment greatly reduces cis-DDP-induced DNA fragmentation, providing additional evidence that LPA and EGF protect ovarian cancer cells from cis-DDP-induced apoptosis.

**DISCUSSION**

After exposure of the human ovarian cancer cell line HEY to cis-DDP, there was a time- and dose-dependent increase in cell death. cis-DDP induces morphological changes, as assessed by light and electron microscopy, associated with PCD, including membrane blebbing and the production of apoptotic bodies. The morphological changes combined with demonstration of DNA release from cells (Table 1) indicate that cis-DDP induces PCD in HEY ovarian cancer cells. In addition to inducing changes in morphology consistent with PCD, cis-DDP also markedly decreased the viability of HEY ovarian cancer cells as indicated by residual colony-forming cell activity (Fig. 1) and MTT dye reduction (Fig. 2). Strikingly, both lipid and peptide growth factors as represented by LPA and EGF induced a marked decrease in the ability of cis-DDP to kill ovarian cancer cells by the process of apoptosis. Because the duration of incubation with LPA or EGF was short and LPA or EGF alone did not alter the proliferative rate of HEY cells in the presence of 10% FBS, LPA and EGF likely directly prevent PCD induced by cis-DDP rather than increase the proliferative rate of the cells.

The mechanism by which LPA and EGF prevent cis-DDP-induced apoptosis remains unclear. Both LPA and EGF induce tyrosine phosphorylation and activate the RAS-mitogen-activated protein kinase (MAPK) kinase signaling pathway, which may prevent PCD. Indeed, it has been shown previously that protein tyrosine kinase inhibitors induce apoptosis in other cell lineages (52). DNA-damaging agents such as cis-DDP can induce an up-regulation of expression of the EGFR (53). This response may function to protect cells from the effects of cytotoxic agents by preventing apoptosis. Thus, incubation with EGF prior to cis-DDP treatment may, in fact, enhance the ability of the tumor cell to escape the cytotoxic activity of cis-DDP. Alternatively, LPA has been demonstrated to increase release of EGFR ligands in other cell lineages (54), and we have demonstrated that activation of the EGFR increases release of a growth factor with LPA-like activity from ovarian cancer cells.6 Furthermore, LPA has been demonstrated to rapidly induce phosphorylation and activation of EGFR family members in murine fibroblasts (55) and ovarian cancer cells, a process required for optimal LPA-induced mitogenesis (55). Thus, LPA and EGF could initiate a cascade resulting in increased production of the heterologous growth factor and thus protect cells from the effects of cis-DDP, or alternatively, both EGF and LPA may result in direct activation of the EGFR family of transmembrane receptors. In support of this contention, anti-EGFR monoclonal antibodies can enhance the antitumor activity of cis-DDP (56). Regardless of the mechanism, interruption of the production or action of LPA or of members of the EGFR family of ligands could sensitize ovarian cancer cells to the action of cis-DDP in vivo.

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4 G. B. Mills, unpublished observations.
5 Y. Xu and G. B. Mills, unpublished observations.
The concentrations of LPA utilized in the experiments described herein are similar to those that are present in the ascites of ovarian cancer patients (22) and thus may play a role in the outcome of cis-DDP therapy in ovarian cancer patients. Whether activation of the EGFR by EGF, TGF-α, or other EGFR ligands occurs in vivo in ovarian cancer patients remains controversial. Although several reports have indicated the presence of EGF and TGF-α in ovarian cancer specimens, other groups have been unable to detect TGF-α in ascites from ovarian cancer patients (reviewed in Refs. 18 and 31). The ability of antibodies against the EGFR to decrease the growth of ovarian cancer cells both in vitro and in vivo (21, 22) argues that ovarian cancer cells as indicated by ovarian cancer cell lines are responsive to the effects of endogenously produced EGF, TGF-α, or other EGFR ligands.

An inverse relationship has been found between EGFR expression and expression of the antiapoptotic gene bcl-2 (57) in a number of human breast carcinomas. Indeed, 70% of bcl-2-negative tumors were found to be EGFR positive (57). Thus, EGFR ligands may play a role in down-regulation of bcl-2. However, in preliminary experiments, although bcl-2 is detectable in ovarian cancer cell lines, cis-DDP, EGF, and LPA did not alter bcl-2 expression in HEY ovarian cancer cells. Thus, bcl-2 does not seem to be involved in the protection conferred by EGF or LPA on cis-DDP-induced PCD. We are currently exploring the possibility that EGF and LPA treatment of ovarian

Fig. 3  LPA and EGF protect the cells from cis-DDP-induced PCD as assessed by light microscopy. Cells were incubated for 24 h with and without 50 μM cis-DDP in the presence or absence of LPA (10 μM) or EGF (25 ng/ml). A, control cells cultured in RPMI supplemented with 10% FBS. B, 50 μM cis-DDP treatment, where many cells have undergone apoptosis. Arrows, cells with typical apoptotic morphology: including membrane blebbing. C, cells treated with 10 μM LPA + 50 μM cis-DDP show significantly increased viability compared to the cis-DDP-treated cells. A smaller percentage of the cells have undergone apoptosis. D, cells exposed to 25 ng/ml EGF + 50 μM cis-DDP also show a significant increase in cell viability, whereby a smaller proportion of cells have undergone PCD, compared to the cis-DDP-treated cells. cis-DDP-induced morphological changes were associated with marked changes in viable cell number, with 100% of control cells being viable (as assessed by trypan blue exclusion). 15% of cis-DDP-treated cells being viable, 44% of cells treated with cis-DDP in the presence of LPA being viable, and 34% of cells treated with cis-DDP in the presence of EGF being viable.
Table 1  EGF and LPA decrease cis-DDP-induced DNA fragmentation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell type</th>
<th>Experiment</th>
<th>% DNA fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>HEY</td>
<td>1</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>8.4</td>
</tr>
<tr>
<td>LPA</td>
<td>HEY</td>
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<td>13.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>9.1</td>
</tr>
<tr>
<td>EGF</td>
<td>HEY</td>
<td>1</td>
<td>18.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>10.8</td>
</tr>
<tr>
<td>cis-DDP</td>
<td>HEY</td>
<td>1</td>
<td>68.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>46.2</td>
</tr>
<tr>
<td>LPA + cis-DDP</td>
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<td>1</td>
<td>9.5</td>
</tr>
<tr>
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<td></td>
<td>2</td>
<td>21.7</td>
</tr>
<tr>
<td>EGF + cis-DDP</td>
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<td>26.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>23.4</td>
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</table>

*The cells in each of these experiments were treated for 24 h with: RPMI supplemented with 10% serum, 10 μM LPA, 25 ng/ml EGF, 50 μM cis-DDP, 10 μM LPA + 50 μM cis-DDP, 25 ng/ml EGF + 50 μM cis-DDP. Percentage of DNA fragmentation was calculated according to the following formula:

\[
\text{DNA fragmentation} = \frac{\text{Fluorescence enhancement of the supernatant}}{\text{Fluorescence enhancement of the pellet harvested}} \times 100 + \text{DAPI staining of DNA as described in "Materials and Methods."}
\]

* Results shown are of two independent experiments showing the averages of two spectrophotometric measurements.

Strategies aimed at altering the production and action of LPA or EGFR have the potential to decrease cis-DDP-induced cell death. Determining which portion of tumors in vivo demonstrates EGF-induced increases in sensitivity or resistance to cis-DDP will require additional studies.

Regardless of the mechanism, both LPA and activation of the EGFR have the potential to decrease cis-DDP-induced cell death of ovarian cancer cells through the process of apoptosis. Strategies aimed at altering the production and action of LPA or ligands for the EGFR thus have the potential to increase the response to cis-DDP and perhaps improve the outcome for this devastating disease.

REFERENCES

Peptide and lipid growth factors decrease cis-diamminedichloroplatinum-induced cell death in human ovarian cancer cells.

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