Increased Sensitivity to cis-Diamminedichloroplatinum(II) in Human Ovarian Carcinoma Cells in Response to Treatment with 12-O-Tetradecanoylphorbol 13-Acetate*

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The tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) enhanced sensitivity to cis-diaminedichloroplatinum(II) (DDP) in human ovarian carcinoma 2008 cells by a factor of 2.53 ± 0.74 fold (S.D.). Sensitization was maximum 3 h after a 1-h exposure to TPA and had disappeared completely by 7 h after treatment. An equivalent degree of sensitization was produced in a 2008 variant selected for 10-fold resistance to DDP. TPA neither increased nor decreased cellular accumulation of DDP. Phorbol, a TPA analog which does not activate protein kinase C, did not cause sensitization. This synergistic interaction between TPA and DDP was completely inhibited by pretreatment with staurosporine, a protein kinase C inhibitor. Cellular cAMP was not altered by TPA stimulation. Furthermore, cycloheximide, a potent protein synthesis inhibitor, did not block the TPA-induced enhancement of drug sensitivity. These results strongly suggest that DDP sensitivity can be modulated by protein kinase C and regulated by phosphorylation of a protein kinase C substrate in both intrinsically sensitive and DDP-resistant cells.

DDP* is one of the most widely used antineoplastic agents for the treatment of human ovarian cancer (1, 2). In spite of its potency, the frequent development of DDP resistance is a major obstacle to curative therapy (3, 4). Although the mechanism of DDP resistance in vivo is not characterized, a good deal of information is available about mechanisms in cell lines. Impairment of DDP uptake is one of the important mechanisms contributing to DDP resistance (5–8). Increased levels of metallothioneins have also been reported in some (9), but not all (10), DDP-resistant cells. Kelley et al. (9) reported that, in a murine leukemia cell line, the degree of resistance was proportional to metallothionein content, and that loss of resistance to DDP in a revertant cell line was associated with concomitant lowering of metallothionein content (9). Modulation of glutathione (GSH) concentrations in mammalian cells has also been reported to influence the cytotoxicity in DDP (11). Some DDP-resistant cell lines contain increased amounts of glutathione (12), and DDP sensitivity can be enhanced under some conditions by extensive depletion of glutathione by buthionine sulfoximine (13). DDP reacts with DNA to produce an intrastrand N7d(GpG)-diammine platinum adduct which comprises 40–60% of the platinum bound to DNA (14). DNA repair defective cells are hypersensitive to DDP (15), and enhanced DNA repair has been implicated in the DDP-resistant phenotype (16).

TPA has been reported to alter cellular sensitivity to several kinds of antineoplastic agents. In the human KB carcinoma cells, TPA treatment decreased sensitivity to etoposide and vincristine by 50%, but this effect could not be mimicked by treatment of cells with 1-oleoyl-2-acetylglycerol (OAG), calling into question a role for protein kinase C (23). Posada (24) demonstrated that TPA enhanced the cytotoxic activity of doxorubicin in sarcoma 180 cells. Conversely, down regulation of protein kinase C produced by long term exposure to TPA resulted in a decreased cytotoxic effect of doxorubicin (24). Protein kinase C can phosphorylate the MDR 1 gene product, which functions as efflux pump for etoposide, vincristine, and doxorubicin. Hofmann et al. (25) reported that either inhibition of protein kinase C activity with a series of compounds such as quercetin (3,3',4',5,7-pentahydroxyflavone), tamoxifen, staurosporine, either lipid analog (BM41440) (26), or down regulation with long term exposure to TPA enhanced the sensitivity of cells to DDP.

We now present evidence that, in contrast to the report of Hofmann et al. (25, 26), TPA increases the sensitivity in cultured human ovarian carcinoma cells to DDP and produces this effect via protein kinase C. This effect occurs in the absence of a change in cellular accumulation of DDP and thus implicates a phosphoprotein as a major determinant of the activity of DDP once it has entered the cell.

MATERIALS AND METHODS2

RESULTS

Effect of TPA on DDP Sensitivity—Fig. 1 shows that when 2008 cells were exposed concurrently for 1 h to 0.1 μM TPA and DDP, TPA increased DDP sensitivity. The IC50 in the

* Portions of this paper (including "Materials and Methods," Table 1, and Figs. 2, 3, and 6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
absence of TPA was 3.07 ± 0.6 μM (S.D.), whereas in the presence of TPA it was 1.2 ± 0.4 μM (S.D.). Thus, TPA produced a 2.5-fold increase in sensitivity. When cells were treated with a 24-h exposure to 0.1 μM TPA followed by a 1-h exposure to DDP, a similar degree of sensitization was observed. However, when cells were treated with continuous exposure to both the TPA and DDP for the full period of colony formation, no change in DDP sensitivity was evident. TPA at concentrations even up to 1 μM did not cause any toxicity by itself; thus, the interaction between TPA and DDP is truly synergistic as defined by median effect analysis (32).

**Time Course of Sensitization**—Ovarian carcinoma 2008 cells were exposed to 0.1 μM TPA and 2 μM DDP either concurrently or with increasing time intervals between the start of 1-h TPA and DDP exposures. Fig. 2 shows that 2 μM DDP reduced colony survival to approximately 80% in the absence of TPA. TPA sensitization was maximal between 1 and 2 h after the start of TPA exposure, but had largely disappeared by 7 h. This time course is consistent with that for activation and inactivation of protein kinase C in other cell systems (23).

**Effect of TPA on Cellular Accumulation of DDP**—2008 cells were treated concurrently with [195Pt]DDP and TPA for 60 min. A vehicle control consisting of an appropriate dilution of acetone was run concurrently. Panel A in Fig. 3 shows that neither the acetone nor the TPA produced any effect on cellular accumulation of [195Pt]DDP. Cells treated with media alone contained 140 ± 13 pmol/mg of protein, whereas those treated with the acetone control contained 141 ± 7 pmol/mg of protein, and those treated with TPA contained 143 ± 21 pmol/mg of protein. Similar experiments were conducted using either a 10-min TPA preincubation followed by a 1-h concurrent TPA and [195Pt]DDP exposure, or a 24-h preincubation with TPA, and none of them showed any effect on DDP accumulation (data not shown). Thus, TPA altered sensitivity by a mechanism which does not involve an increased amount of drug entering the cell.

**Effect of TPA on Cellular cAMP Content**—The cytotoxicity of DDP can be increased by elevating the cAMP level, indicating that protein kinase A activity is a determinant of DDP sensitivity (33). To determine whether TPA was enhancing sensitivity via this mechanism, cellular cAMP levels were determined at 10 and 60 min following the start of exposure to 0.1 μM TPA. Forskolin, a potent activator of adenylate cyclase (34), was included as a positive control. The data presented in Table 1 shows that the acetone vehicle control reduced cAMP content to 59% of the untreated control. TPA reduced the cAMP level to 38% of the untreated control or 65% of the vehicle control at 60 min. In contrast, forskolin increased the cAMP content by 297-fold. Although the biological significance of the TPA-induced reduction in cAMP level is not clear, TPA clearly did not increase cAMP levels into the range associated with forskolin-induced enhancement of DDP sensitivity (33).

**Effect of Phorbol on DDP Sensitivity**—The results presented above suggested that protein kinase C may be involved in regulating DDP sensitivity. To test this further, cells were treated with phorbol, a compound that has structural homology to TPA but not to diacylglycerol, and is known to be inactive as a tumor promoter and activator of protein kinase C. As shown in Fig. 4, phorbol failed to enhance DDP sensitivity, and in fact slightly reduced it.

**Effect of Protein Kinase C Inhibitors on TPA Enhancement of DDP Sensitivity**—Staurosporine was used as a potent inhibitor of protein kinase C activity (35). 2008 cells were treated with TPA and DDP either in the presence or absence of 5 nM staurosporine for 1 h. DDP (2 μM) alone reduced survival to 85 ± 5% of control, whereas the addition of 0.1 μM TPA enhanced sensitivity reducing survival to 38 ± 16% of control. In Fig. 5, these data have been normalized such that the survival with 2 μM DDP alone is 100%, and the addition of TPA reduced survival to 44%. When the cells were pretreated with staurosporine, the effect of TPA was completely blocked, and survival in the presence of all three agents was 96% of that produced by DDP alone. Control experiments showed that staurosporine by itself did not enhance or inhibit sensitivity to DDP. These data strongly suggest that TPA sensitization is mediated by protein kinase C.

**Enhancement of Sensitivity in DDP-resistant Cells**—The 2008/C13*5 cell line is 8-10-fold resistant to DDP. Fig. 6 shows that when these cells were exposed for 1 h concurrently to TPA and DDP, the IC50 was reduced from 13.1 ± 2.7 μM (S.D.) to 4.9 ± 1.4 μM (S.D.). This 2.6-fold sensitization was nearly identical with the 2.5-fold sensitization observed in the DDP-sensitive 2008 cells. At the end of a 1-h incubation, the DDP-resistant 2008/C13*5 cells had accumulated 37.0 ± 3 pmol/mg of protein in cells treated with media alone, 38.7 ±
by more than treatment did CHM for 10 min was sufficient to inhibit protein synthesis or absence (0) of low

FIG. 4. Effect of phorbol on DDP sensitivity. Cells were treated with appropriate concentrations of DDP in the presence (●) or absence (C) of 10⁻⁷ M phorbol for 1 h. Drug cytotoxicity was determined by colonogenic assay on plastic dishes. Points, mean values of three experiments performed with triplicate cultures; bars, S.D.

3 pmol/mg of protein in the acetone vehicle-treated cells, and 33.0 ± 2 pmol/mg of protein in the TPA-treated cells.

Effect of Protein Synthesis Inhibition—Preliminary experiments showed that incubation of 2008 cells with 5 μg/ml CHM for 10 min was sufficient to inhibit protein synthesis by more than 50% (data not shown). Nevertheless, such treatment did not have any effect on TPA chemosensitization to DDP. TPA reduced survival to 33 ± 5% (S.D.) of control in the absence of CHM, and 41 ± 7% (S.D.) of control in the presence of CHM. Thus, induction of DDP sensitivity was neither blocked nor augmented by CHM, suggesting that TPA-induced changes occurred in the absence of protein synthesis.

DISCUSSION

The results presented in this paper provide four lines of evidence arguing that protein kinase C is involved in the regulation of DDP sensitivity. First, TPA produced a consistent increase in DDP sensitivity under conditions where, even at a 10-fold higher concentration, it was totally nontoxic. Although TPA may do other things as well (17–20), the major effect by which it mediates changes in cell phenotype appears to be through the activation of protein kinase C. Second, phorbol, an inactive analog of TPA, was unable to enhance DDP sensitivity. Third, the TPA-induced increase in DDP sensitivity was completely blocked by pretreatment of the cells with staurosporine for 1 h. The cogency of this point is somewhat diluted by the fact that staurosporine can produce a variety of other effects as well (35). Fourth, the time course of sensitization is consistent with the expected time course for activation or inactivation of protein kinase C following a 1-h TPA exposure (23). Enhancement was apparent after a 1-h concurrent exposure, was maximal 2 h from the start of TPA exposure, and had disappeared by 7 h. Taken together, these results provide very strong evidence for the involvement of protein kinase C.

Prolonged exposure of cells to TPA produces down regulation of protein kinase C activity in a variety of cell systems (23), and if it is stimulation of protein kinase C activity that accounts for enhanced DDP sensitivity, one might have expected a 24-h exposure to TPA to reduce DDP sensitivity. Instead, a 24-h exposure to TPA produced exactly the same degree of enhancement as a 1-h exposure. Protein kinase C activity was not measured at the end of the 24-h exposure, and it is possible that the enzyme was not down regulated in these cells as occurs also in KB cells (23). The protein(s) phosphorylated by protein kinase C that transmit the signal for enhanced DDP sensitivity is not known. We have previously reported that activation of adenyl cyclase with forskolin, or elevation of cAMP with dibutyl cAMP, enhances sensitivity to DDP in a synergistic manner (33). Wiener and Scarpa (36) reported that TPA could potentiate the forskolin-induced cAMP response, and Plet et al. (37) showed that TPA increased protein kinase C activity. However, several lines of evidence argue against involvement of protein kinase A in the TPA-mediated enhancement of DDP sensitivity. First, direct measurement of cAMP showed no change when cells were treated with TPA. Second, activation of protein kinase A by forskolin is associated with enhanced DDP uptake into the cell, an effect not produced by TPA. Third, TPA was equally effective in the DDP-sensitive 2008 cells and the DDP-resistant 2008/C13*5 cells. In contrast, the ability of forskolin to enhance DDP sensitivity is markedly blunted in 2008/C13*5 cells compared to the 2008 cells. These points also argue for the involvement of two separate protein kinases in the regulation of DDP sensitivity.

TPA enhancement of DDP sensitivity was associated with no change in cellular DDP accumulation. Since the cytotoxicity of DDP is believed to be related to the extent of DNA intrastrand, interstrand, and DNA protein cross-link formation, the results suggest that intracellular drug is more effective at platinating DNA following TPA treatment. Three mechanisms of DDP resistance have been identified which
can participate in this: decreased conjugation with glutathione, decreased binding to metallothioneins, or decreased DNA repair. The human metallothionein II gene is one of the TPA-inducible genes (38, 39) and, in contrast to what was observed, an increase in the transcription rate of the metallothionein II* gene might be expected to result in DDP resistance. However, it is conceivable that TPA is altering the ability of metallothioneins to bind DDP, glutathione levels, glutathione-S-transferase activity, or DNA repair activity. The fact that the TPA effect was equivalent in DDP-sensitive and -resistant cells indicates that selection for DDP resistance did not produce any lesions in the TPA signal transduction pathway involved in this sensitization. The signal pathway remains fully intact in both types of cells, indicating that the changes that account for DDP resistance involve biochemical steps distinct from those that participate in the TPA signal transduction pathway.

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REFERENCES

Potentiation of Cisplatin Sensitivity

Table 1: Effect of TPA and Arachidonic Acid on PAF Accumulation

<table>
<thead>
<tr>
<th>Duration of Treatment</th>
<th>Treatment</th>
<th>Mean ± SD</th>
<th>Mean ± SD</th>
</tr>
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<tr>
<td>10 min</td>
<td>No treatment</td>
<td>1.9 ± 1.9</td>
<td>21.4 ± 18.6</td>
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<tr>
<td></td>
<td>Acetone</td>
<td>0.8 ± 1.9</td>
<td>5.6 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>TPA</td>
<td>2.7 ± 1.9</td>
<td>8.2 ± 2.8</td>
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<tr>
<td>60 min</td>
<td>No treatment</td>
<td>21.5 ± 30.6</td>
<td>94.8 ± 40.5</td>
</tr>
</tbody>
</table>

a: p<0.01 vs control. b: significant interaction of drug treatment on PAF accumulation at the p<0.01 level, as determined by the Student's t test.

Fig. 1. Time course of change in PAF sensitivity of 293 cells. Cells were pre-treated with 0.1 μM DDP alone or in the presence of TPA for 6 h. The cells were harvested at 0, 20, 30, 40, and 50 h, and the PAF accumulation was determined by ELISA. Each point represents the mean ± SEM of at least 3 experiments.

Fig. 2. Accumulation of [3H]PAF in 293 cells treated with TPA and/or DDP. Cells were treated with [3H]PAF (20 μM) alone or in the presence of TPA (1 μM) and/or DDP (10 μM). Data are expressed as mean ± SEM. Each point represents the mean ± SEM of at least 3 experiments.
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